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Inflammation and Skin Cancer Mediated Through c-Fos/AP-1

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As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon—don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians, Cyclops,
wild Poseidon—you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbors you're seeing for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind—
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.

Ithaka. *KP Kavafis*

Dedicated to my family

In memory of my grandmother

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Summary

In this study I describe a novel mechanism by which increased levels of c-Fos/AP-1 transcription factor in the epidermis promotes the development of epidermal preneoplastic lesions and eventually, upon 7,12-dimethyl-benz[a]anthracene (DMBA) treatment, it contributes to the development of skin Squamous Cell Carcinomas (SCCs). To unravel the function of epidermal c-Fos, we have generated a mouse model (c-Fos$^{Ep\text{-}tetON}$) in which we can inducibly activate the expression of *c-fos* in the basal layer of the epidermis as well as in other stratified epithelia. I show that inducible *c-fos* expression triggers innate and adaptive immune responses in the epidermis, particularly, transient recruitment of Gr1^{+} cells and chronic recruitment of CD4 T lymphocytes. In addition, broad genome-wide expression analyses identified two direct and novel transcriptional target genes of c-Fos, *mmp10* and *s100a7a15*. Both target genes are involved in inflammatory processes, being able to recruit Gr1^{+} cells and CD4 T lymphocytes, as well as in the development of cutaneous cancers. Importantly, using a broad matrix metalloprotease (MMP) inhibitor to reduce MMP10 activity in the epidermis, we observed amelioration of the development of the preneoplastic lesions of the skin. Furthermore, in the absence of mature B and T cells in a Rag1-deficient background, a drastic improvement of the disease in Fos$^{Ep\text{-}tetON}$ mice was observed. Moreover, upon DMBA-induced H-Ras mutations, c-Fos is sufficient to promote the development of skin SCCs. Interstingly, in this setting, tumor size, number and burden were significantly reduced upon blockade of inflammatory responses by means of a cyclooxygenase 1 and 2 (COX-1/COX-2) inhibitory treatment following DMBA treatment and inducible expression of *c-fos*. Finally, I have seen a strong correlation between human c-FOS and MMP10 expression in human SCCs, where they are abundantly expressed, compared to BCCs, where no expression of these two proteins is observed. In addition, a strong correlation between c-FOS expression and CD4 T cell infiltrates were observed in human SCCs. This thesis has identified two novel and direct transcriptional targets of c-Fos, MMP10 and S100a7a15, clearly involved in mediating CD4 T cell-mediated immune responses in the epidermis and thereby contributing to the development of preneoplastic lesions, which upon oncogenic insults, eventually develop into SCCs. Here I propose two new candidate proteins that could be of therapeutic interest to treating cutaneous SCCs.

Resumen

Los resultados de mi proyecto de tesis han dado lugar a la descripción de un nuevo mecanismo mediante el cual el factor de transcripción c-Fos/AP-1 promueve el desarrollo de lesiones preneoplásicas en la epidermis y en combinación con la aplicación de DMBA, promueve el desarrollo de carcinomas epidermoides (SCCs). Para estudiar la función de c-Fos en la epidermis, hemos generado un modelo genético de ratón (c-Fos$^{Ep\text{-}tetON}$) en el cual se puede inducir la expresión de *c-fos* en la capa basal de la epidermis y en otros epitelios estratificados. Aquí demuestro que la expresión inducible de *c-fos* promueve respuestas inmunes innatas y también adaptativas en la piel, fomentando la infiltración de células Gr1$^+$ transitoriamente y de linfocitos T CD4$^+$ de forma crónica. Mediante análisis de expresión genómica se identificaron dos nuevos genes diana, *mmp10* y *s100a7a15*, ambos implicados en procesos inflamatorios así como también en cánceres de piel. La inhibición de metalloproteasas (MMPs) mediante el uso de un inhibidor, redujo significativamente la progresión de las lesiones preneoplásicas tras la inducción de la expresión de *c-fos* en ratones c-Fos$^{Ep\text{-}tetON}$. Además, una mejora drástica se observó en el 80% de los casos al deplecionar las poblaciones de linfocitos T y B maduros, mediante el uso de un ratón knock-out para Rag1. Asimismo, en esta tesis he demostrado que el factor de transcripción c-Fos/AP-1 es suficiente para promover el desarrollo de carcinomas epidermoides en ratones tratados con el carcinógeno DMBA. En este mismo sistema, el bloqueo de procesos inflamatorios en la piel, mediante la inhibición de las enzimas COX1 y COX2, en ratones inducidos con DMBA y c-Fos se observa una disminución en el desarrollo de carcinomas epidermoides y aquellos desarrollados son más pequeños. Finalmente, los análisis de muestras humanas de carcinomas epidermoides y carcinomas basales de piel han descubierto la posible implicación de c-FOS sólo en los carcinomas epidermoides dado que el 80% de estos tumores presentaba altos niveles de c-FOS, mientras que los carcinomas basales no lo expresaban. Finalmente, he observado una correlación entre la expresion de c-FOS y de MMP10 en carcinomas epidermoides, MMP10 podría perfectamente ser un gen diana de c-FOS en humano como observamos en ratón. Asimismo, se observó correlación entre los niveles de expresión de c-FOS en los carcinomas epidermoides y los altos niveles de infiltración de células T CD4$^+$. Esta tesis ha servido para identificar dos genes transcripcionalmente activados por c-Fos, *mmp10* y *s100a7a15,* claramente implicados en el desarrollo de procesos inflamatorios mediados por células T CD4$^+$ en la piel y que

promueven inicialmente el desarrollo de lesiones preneoplásicas y eventualmente el desarrollo de carcinomas epidermoides. El desarrollo de fármacos específicos para estas dos dianas puede ser de uso terapéutico en carcinomas epidermoides.

Abbreviations

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metallopreinase with thrombospoindin motifs
AP-1	Activator Protein 1
ATF	Activating transcription factor
BCC	Basal Cell Carcinoma
BSA	Bovine Serum Albumine
ChIP	Chromatin IP
CTLS	Cytotoxic T lymphocytes
DC	Dendritic cell
DMBA	7,12-dimethyl-benz[a]anthracene
Dox	Doxycycline
ECM	Extracellular matrix
EdU	5-ethynyl-2`-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GM-CSF	Granulocyte monocyte colony stimulating factor
HEK	Human epidermal keratinocytes
HF	Hair follicle
HPV	Human papillomavirus
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
IFE	Interfollicular epidermis
IHC	Immunohistochemistry
IP	Intraperitoneal
JNK	Jun N-terminal kinase
K1	Keratin 1
K10	Keratin 10
K5	Keratin5
K6	Keratin 6
KGF	Keratinocyte growth factor
LOH	Loss of heterozigosity
LOX	Lysil oxidase
MAPK	Mitogen-activated protein kinase
MMP	Matrix Metalloprotease
NK	Natural Killer
NMSC	Non melanoma skin cancer
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PTCH1	Patched1
RT	Room temperature

RtTA	Reverse transactivator
SC	Stem cell
SCC	Squamous Cell Carcinoma
SDS	Sodium dodecyl sulfate
SHH	Sonic Hedgehog
SLE	Systemic Lupus Erythematosus
SMO	Smoothened
SOS	Son of Seveless
TACE	TNFa-Converting Enzyme
Tet	Tetracycline
TEWL	Transepidermal Water Loss
Tgf-β	Transforming Growth Factor Beta
TNFα	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoylphorbol-13-acetate
tTA	Transactivator
VEGF	Vascular endothelial growth factor

Introduction

1. THE SKIN

The skin is the body´s largest organ. It serves as a protective barrier that protects against loss of fluids, physical trauma and invasion by harmful microbes. The skin is divided into two different compartments, the dermis and the epidermis. Interactions between epithelial and mesenchymal cells play a crucial role in the regulation of tissue morphogenesis, homeostasis and repair (Beck and Blanpain, 2012).

1.1. The Dermis

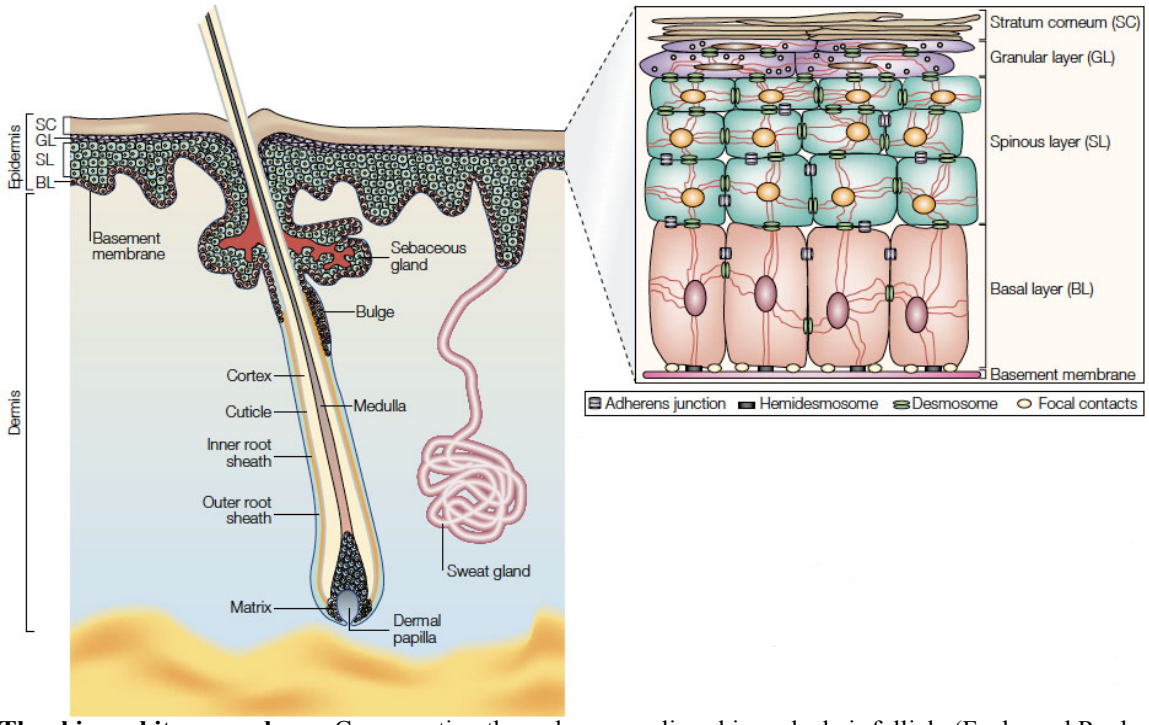
The dermis is the fibrous connective tissue between the epidermis and the subcutaneous fat and is responsible for providing nutrients and physical support to the epidermis (Burr and Penzer, 2005). The most abundant cell type in the dermis is the fibroblast (McLafferty et al., 2012). Fibroblasts synthesize the extracellular matrix and collagen required for normal homeostasis. Type I collagen is by far the most abundant protein in human skin, comprising greater than 90% of its dry weight and the unique physical properties of collagen fibers is to confer structural integrity to skin (Fisher et al., 2008). The dermis also contains blood and lymph vessels, nerve endings, hair follicles and glands. Immune cells also populate the dermis. Some immune cells like mastocytes, histiocytes and γ/δ T lymphocytes are resident in the skin, but other leukocytes like T cells migrate to the skin upon injury or infection (Gebhardt et al., 2011).

1.2. The Epidermis

The epidermis is a stratified epithelium that contains a single inner (basal) layer of proliferative keratinocytes that adhere to the basement membrane, which is rich in extracellular matrix and growth factors, and separates the epidermis from the underlying dermis. Cells in the basal layer are responsible for generating the layers of non-dividing cells that undergo a program of terminal differentiation as they move outward and are continually shed from the skin surface (Figure 1) (Fuchs and Nowak, 2008). The balance between proliferation and differentiation is tightly regulated, since the disruption of this balance causes several pathological conditions including inflammation and tumorigenesis (Blanpain and Fuchs, 2009). Indeed, a disruption of the differentiation-promoting Notch signaling pathway or a hyperactivation of the EGFR-

Ras-MAPK signaling pathway in keratinocytes, leads to the development of epithelial tumors (Demehri et al., 2009; Brown et al., 1998).

Figure I1.



The skin and its appendages. Cross-section through mammalian skin and a hair follicle (Fuchs and Raghavan, 2002)

As epidermal keratinocytes exit the basal layer and cease to proliferate, they progress upward through three distinct differentiation stages: spinous layer, granular layer and *stratum corneum*. The major structural change at the basal-to-spinous-layer transition is the switch from keratin 5 and keratin 14 intermediate filaments in the basal layer to Keratin 1 and Keratin 10 suprabasally. Additional changes occur in the basal/spinous transition, such as downregulation of p63, a member of the p53 family of transcription factors (Dotto, 2009). p63 is expressed in basal cells of all stratified epithelia and is thought to represent a master regulator of the stratification process (Blanpain et al., 2007). Indeed, p63-deficient mice die postnatally from severe developmental anomalies, including a lack of stratified epithelia (Mills et al., 1999).

As cells enter the granular layer, the primary cornified envelope protein loricrin is expressed and lamellar granules packed full of lipids appear (Blanpain and Fuchs, 2009). Profilaggrin is also expressed at this time, and soon afterwards, it is proteolytically processed to generate filaggrin, a protein that bundles keratin filaments

into indestructible cables (Aho et al., 2012). As granular cells transit to the *stratum corneum*, the metabolical activity ceases, and an influx of calcium results in activation of transglutaminases, that initiate glutamyl-e-lysine crosslinks to produce the cornified envelope, characteristic of this layer (Eckert et al., 2005). The cornified envelope surrounds cells in the *stratum corneum* and contributes to the skin's barrier function (Simpson et al., 2011). Eventually, keratinocytes in the stratum corneum undergo apoptosis and are released from the surface of the epidermis (Blanpain and Fuchs, 2009).

The epidermis also has the remarkable ability to elaborate the body surface with appendages, which range from hair follicles, nails, oil and sweat glands in mammals to scales and feather in lower vertebrates (Fuchs and Nowak, 2008).

Besides keratinocytes, three other cell types are found in the epidermis: melanocytes, Langerhans cells and Merkel cells. They are not abundant, but have important functions. Melanocytes are located in the lower part of the epidermis and they synthesize melanin, the pigment that gives skin the natural color (Haass and Herlyn, 2005). Langerhans cells are dendritic cells (antigen-presenting immune cells) of the epidermis and they are present in all layers of the epidermis, but are most prominent in the *stratum spinosum* where they take up and process microbial antigens to become fully functional antigen presenting cells (Romani et al., 2012). Merkel cells are oval receptor cells that have synaptic contacts with somatosensory neurons and these cells are involved in the sensation of light touch (Boulais and Misery, 2007).

1.3. Epidermal and dermal crosstalk

Interactions between mesenchymal and epithelial cells are responsible for complex events such as tissue development, homeostasis and repair (Werner and Smola, 2001). This mutual crosstalk mainly involves growth factors and cytokines. Pioneering studies of Rheinwald and Green demonstrated that normal human epidermal keratinocytes depend on the presence of fibroblasts for efficient growth in tissue culture (Rheinwald and Green, 1975). Later on Rubin, *et al* identified a fibroblast-derived growth factor (FGF), termed Keratinocyte Growth Factor (KGF), that strongly stimulated the proliferation of keratinocytes (Rubin et al., 1989). Moreover, the group of Peter Angel described a paracrine loop in which by using immortalized fibroblasts deficient

for c-Jun and JunB, in combination with human primary keratinocytes in the three-dimensional organotypic co-culture system, observed that the lack of either one of these transcription factors in fibroblasts severely affected proliferation and differentiation of the overlying normal human keratinocytes (Szabowski et al., 2000). This was due to a direct transcriptional regulation of KGF and GM-CSF by Jun/AP-1 proteins in fibroblasts. Furthermore, this crosstalk could also have an impact in epithelial tumorigenesis. Indeed, recent studies have shown that a disruption in the Notch signaling pathway in the mesenchymal compartment leads to the secretion of soluble factors in an AP-1 dependent manner (FGF7, FGF10, CSF1, MMP3 and MMP13). These factors create an appropriate microenvironment in the dermis, also termed as "field cancerization", that promotes epithelial tumorigenesis (Hu et al., 2012).

1.4. Epidermal Stem Cells

The adult epidermis and its appendages undergo continuous renewal and maintain reservoirs of multipotent stem cells (SC). Different stem cell pools have been found in the hair follicle (HF) as well as in the interfollicular epidermis (IFE).

HF stem cells reside in a specialized microenvironment called the bulge. These cells cycle slowly, as revealed by their ability to retain a pulse of nucleotide label following weeks of chase (Alonso and Fuchs, 2003). The bulge is composed of a heterogeneous population of self-renewing multipotent cells. Stem cell subpopulations in the bulge exhibit different locations (basal versus suprabasal) and different characteristics, like slowly cycling (quiescent) versus rapidly cycling (Fuchs, 2009). In contrast to the HF, much less is known about stem cells in the IFE. Although lineage-tracing experiments have clearly demonstrated that homeostasis in mouse epidermis is fueled by an independent stem cell population, their origin and localization is still a matter of debate (Beck and Blanpain, 2012).

Stem cells in the basal layer of the epidermis can undergo symmetric and asymmetric cell division. The maintenance of a constant pool of stem cells can be accomplished by one of two distinct types of cell divisions during tissue homeostasis: in asymmetric division, where one daughter remains a SC throughout self-renewal, and the other daughter becomes committed to enter a program of terminal differentiation. By contrast, symmetrical cell divisions result in both daughter cells adopting the same fate,

which for SCs would result in the generation of two SCs (self-renewal) or two differentiated cells (symmetric differentiation).

1.5. Human skin versus mouse skin

The stratification of mouse skin and human skin is similar—although distinct differences do exist, such as the increased thickness of human skin in comparison with that of mice (Lowes et al., 2007). Mouse skin is heavily populated by hair follicles, whereas the human epidermis is mainly interfollicular; the differences are less striking in skin from the ear and the tail of mice compared with the hairy back skin. Moreover, mouse skin lacks sweat glands and melanocytes in the interfollicular epidermis, but in contrast shows a synchronized hair cycle, rapid epidermal turnover and the presence of intra epidermal γδ T cells (Berking et al., 2002; Jameson et al., 2004; Khavari, 2006; Wagner et al., 2010). The murine immune system contains particular subtypes of cells, including $CD8^+$ dendritic cells (DCs), dendritic epidermal T cells and natural killer (NK) 1.1^+ T cells that are absent in human skin. Despite the obvious differences between mouse skin and human skin, mouse models have been successfully employed to mimic human skin disease in contact hypersensitivity, wound healing, inflammation as well as skin cancer to model monogenic hereditary skin diseases. Clearly, mouse and human skin have many aspects and molecular pathways in common.

2. SKIN CANCER

Skin cancer is the third most common human malignancy and its occurrence has been increasing rapidly over the past decades. An estimated number of 2-3 million non-melanoma skin cancer patients and 132,000 patients of melanoma are counted every year (World Health Organization).

Melanoma is the type of skin cancer that arises from the melanocytes, melanin-producing cells located in the basal layer of the epidermis. It is the most dangerous type of skin cancer as it is the leading cause of death from skin disease (Tsao et al., 2012). 10% of melanoma patients have a family history that confers approximately a twofold increase in probability to develop melanoma. Several genes have been identified to predispose to melanoma. Amongst others, mutations in *CDKN2A* (encoding p16), *CDK4,*

RB1 lead to the development of melanoma. Other genes such as PTEN or B-RAF have been described to promote melanoma (Maubec et al., 2012; Rezze et al., 2012).

Non-Melanoma Skin cancer comprises two major types of Skin Cancers, Basal and Squamous Cell Carcinoma (BCC and SCC). Both tumors arise from keratinocytes, but are very different in morphology and in the underlying mechanisms (Colmont et al., 2012).

2.1. Basal Cell Carcinoma (BCC)

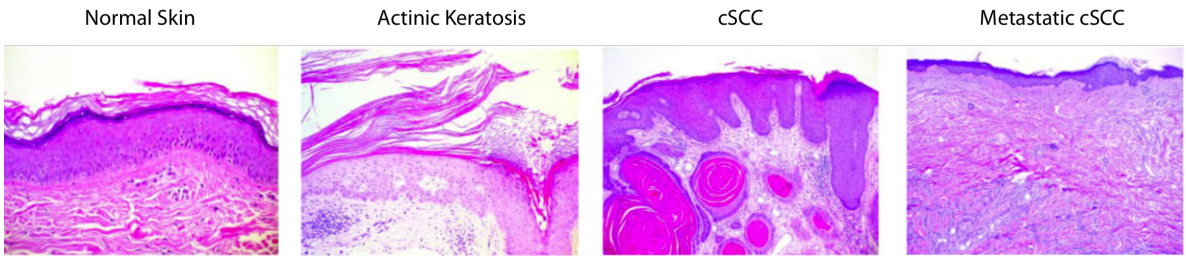
It is the most common and least dangerous form of skin cancer (Kasper et al., 2012). It grows slowly, usually on the head, neck and upper torso. BCCs appear on skin exposed to UV light radiation and typically occur in the fourth decade of life and beyond (Kasper et al., 2012). Pathologically, it resembles the keratinocytes in the basal layer of the epidermis (Crowson, 2006). The vast majority of BCCs occur sporadically, but patients with the rare heritable disorder "Basal cell nevus syndrome" have a marked susceptibility to developing BCCs. Several genes of the Sonic Hedgehog (SHH) signaling pathway are frequently mutated (Epstein, 2008). Approximately 90% of sporadic BCCs have identifiable mutations in at least one allele of *PTCH1,* and an additional 10% have activating mutations in the downstream effector, smoothened (SMO) protein, which renders SMO resistant to inhibition by PTCH1 (Epstein, 2008). Several compounds targeting members of the SHH signaling pathway are being used in clinical trials (Kasper et al., 2012). Removal of tumors using surgery is widely established in less invasive BCCs.

2.2. Cutaneous Squamous Cell Carcinoma (SCC)

Cutaneous Squamous cell carcinoma is the second most common type of human cancer with over 250,000 new cases annually in the USA and it is the second in incidence after BCC. It arises from keratinocytes of the epidermis and oral mucosa. Pathologically, keratinocytes in this type of cancer share features with the squamous cells seen in the outermost layers of the epidermis. Unlike BCCs, cutaneous SCCs are associated with a substantial risk of metastasis (Ratushny et al., 2012). SCC is most commonly found in sun-exposed areas. Besides ultraviolet light, other risk factors have been associated with skin SCC, such as arsenic exposure, tobacco and human papilloma virus infection (Brantsch et al., 2008). SCC typically manifests as a spectrum of progressively advanced

malignancies, ranging from a precursor lesion like actinic keratosis to SCC "in situ", invasive SCC and finally metastatic SCC (Figure 4) (Ratushny et al., 2012).

Figure I2.



Histological features of the different stages of human SCC development, from healthy skin to metastatic SCC.

Several syndromes like Xeroderma Pigmentosum or Epidermolysis bullosa have been associated with increased risk to develop SCCs. As with other cancers, SCCs exhibit impaired genomic maintenance that facilitates acquisition of new mutations. *p53* is commonly mutated in dysplastic lesions. 40% of SCC *in situ* harbors p53 mutations, indicating that p53 loss occurrs prior to tumor invasion (Campbell et al., 1993). Aberrant activation of EGFR (Epidermal Growth Factor) and Fyn, a Src-family tyrosine kinase, are seen in human SCCs. Furthermore, amplification and activating mutations of the *Ras* oncogene have been found in SCCs (Pierceall et al., 1991). 21% of SCCs harbor activating *Ras* mutations. Of the three *Ras* genes, Harvey rat sarcoma virus oncogene (*H-Ras*) is preferentially mutated in SCCs.

Skin SCC has been extensively modeled by either making use of genetically modified mice, such as K14-HPV or the K5-SOS mouse models (Arbeit et al., 1994; Sibilia et al., 2000), or by using the two-step chemical carcinogenesis protocol (Kemp, 2005). In this protocol, mutations in *H-Ras* are induced by a single topical dose of a carcinogen, most commonly 7,12-dimethyl-benz[a]anthracene (DMBA), applied on the back skin. Repeated topical applications of a tumor promoter, such as TPA give rise to benign neoplastic lesions, which causes sustained hyperplasia (papillomas) and inflammation. A small percentage of these papillomas progress to malignant invasive squamous cell carcinomas (SCC). In carcinomas, loss of heterozygosity (LOH) and mutations of the tumor suppressor p53 are frequent. More aggressive carcinomas show additional LOH and mutations of the tumor suppressors *p19/Arf* and $p16^{Ink4a}$ (Kemp, 2005). The use of

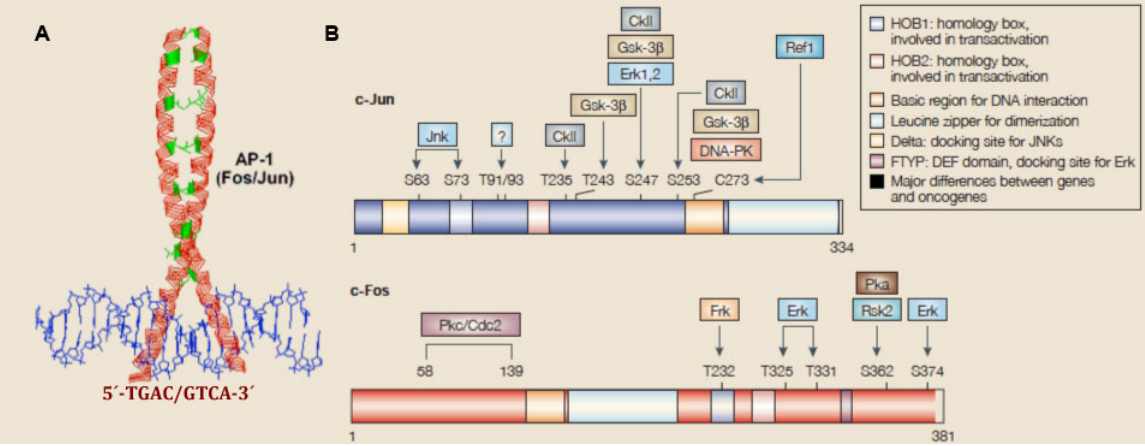
these mouse models has allowed extensive analyses on the underlying mechanisms of cutaneous SCC development

3. THE ACTIVATOR PROTEIN 1 (AP-1) TRANSCRIPTION FACTOR

3.1. Structure and function

The transcription factor activator protein 1 (AP-1) consists of dimers composed of members of the Jun and Fos, as well as activating transcription factor (ATF) and MAF protein families. In contrast to the Fos and Fos-related proteins (Fos, FosB, Fra-1 and Fra-1), which can only heterodimerize with members of the Jun family, Jun family members (Jun, JunB and JunD) can homodimerize or heterodimerize with Fos members (Jochum et al., 2001). A common feature of all these proteins is the evolutionarily conserved bZIP (basic DNA-binding) domain combined with a leucine zipper region (Figure 2). The leucine zipper is responsible for dimerization, which is a prerequisite for DNA binding mediated by the bZIP domain. AP-1 homo- or heterodimers bind to the classic 12-O-Tetradecanoylphorbol-13-acetate (TPA)-responsive element or TRE with the consensus sequence TGAC/GTCA (Figure 2) (Angel et al., 2001)

Figure I3.



AP-1 transcription factor. A. Schematic respresentation of the AP-dimer binding to a TRE element on the DNA. **B**. Structure of mouse c-Jun and c-Fos proteins. *Adapted from Eferl and Wagner, 2003.*

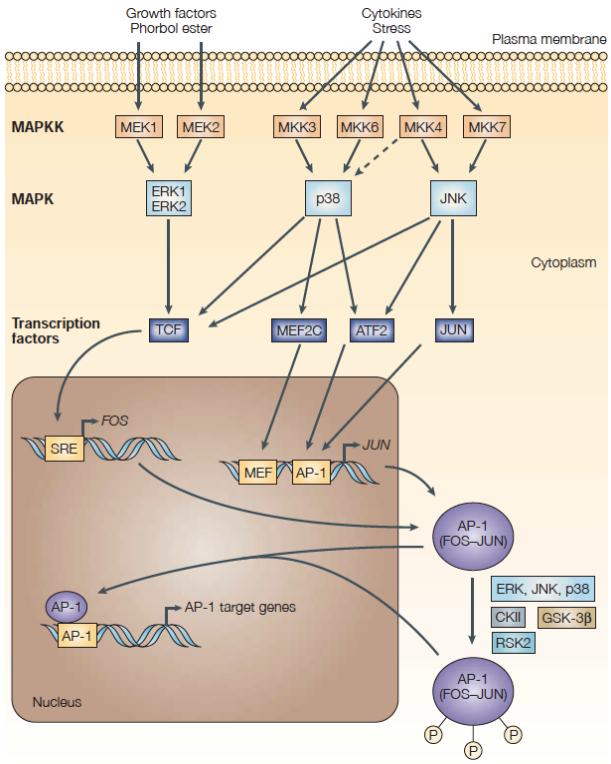
Although members of the Jun and Fos families share high degree of structural homology, the individual AP-1 dimers show significant differences in their DNA binding affinity and their capability to activate or suppress gene expression. The different dimer

combinations recognize different sequence elements in the promoters of target genes and exert different activities.

3.2. AP-1 signal transduction

AP-1 controls distinct biological processes including cellular proliferation, differentiation, oncogenic transformation, apoptosis and inflammation (Zenz et al., 2008). AP-1 activity is regulated in a given cell by a broad range of physiological and pathological stimuli, including growth factors, neurotransmitters, hormones, infections as well as chemical stresses (Eferl and Wagner, 2003). These stimuli activate a complex network of signaling cascades involving MAPK like ERK (Extracellular Signal-Regulated Kinase), p38 stress activated kinase and JNK (Jun Nuclear Kinase). In turn, these activated kinases modulate AP-1 activity by phosphorylating different substrates, including Jun and Fos proteins (Figure 3). ERKs phosphorylate c-Fos, Fra-1 and Fra-2, thereby enhancing their DNA binding activity, as they heterodimerize with c-Jun. JNKs phosphorylate c-Jun very efficiently, JunD less efficiently, but they do not phosphorylate JunB. Effective JNK substrates require a separate docking site and specificity-conferring residues flanking the phosphoacceptor. The docking site increases the efficiency and specificity of the phosphorylation reaction (Kallunki et al., 1996).

Figure I4.



Transcriptional and post-translational modifications of AP-1. *Adapted from Eferl and Wagner, 2003.*

3.3. Biological functions of AP-1 in mice

The use of broad or tissue-specific gain- and loss-of-function mouse models has been instrumental to understand the biological functions of the different AP-1 members in development and disease. Most members of the AP-1 family of transcription factors are essential for embryonic development, since c-Jun, JunB and Fra-1 knock-out fetuses die between E9.5 and E12.5, whereas Fra-2 knock-out pups die soon after birth (Fra-2) (Hilberg et al., 1993; Schorpp-Kistner et al., 1999; Eferl et al., 2007). Studies with knock-out and transgenic mice have assigned distinct functions to c-Fos and Fra-1. Knock-in mice that express Fra-1 in place of c-Fos can rescue c-Fos-dependent functions such as bone development (Fleischmann et al., 2000). Moreover, JunB can substitute for absence of c-Jun during mouse development since JunB can rescue both liver and cardiac defects in c-Jun-null mice (Passegue et al., 2002). For further information regarding specific functions of AP-1 in different tissues see Table S1.

3.3.1. Biological functions of AP-1 in skin

Within the epidermis AP-1 proteins are found expressed in all layers, although with different patterns (Angel et al., 2001). None of the viable single knock-out mice display dramatic skin phenotypes suggesting that individual AP-1 proteins are either dispensable or redundant during skin development (Zenz et al., 2008). However, keratinocyte-specific single deletion of *junB* or combined constitutive or inducible deletion of both *c-jun* and *junB* in the basal layer of the epidermis leads to the development of inflammatory skin diseases, such as Psoriasis (Meixner et al., 2008; Pflegerl et al., 2009; Zenz et al., 2005; Schonthaler et al., 2009). Interestingly, c-Fos seems to be dispensable for epidermal development (Guinea-Viniegra et al., 2012; Saez et al., 1995).

3.3.2. AP-1 functions in tumorigenesis

Jun and Fos proteins were initially identified as the viral oncoproteins v-Fos and v-Jun in the Finkel-Biskis-Jenkins osteosarcoma virus and avian sarcoma virus 17, respectively (Maki et al., 1987; Curran et al., 1983).

A number of the AP-1 proteins, such as c-Fos, FosB and c-Jun, can transform several cell types efficiently in culture and have been linked to cancer (Jochum et al.,

2001)). When widely overexpressed in mice, only some of these proteins can promote tumorigenesis. *c-fos* transgenic mice develop osteosarcomas by the transformation of chondroblasts and osteoblasts (Wang et al., 1991; Grigoriadis et al., 1993). Moreover, c-Fos can also display tumor suppressive functions, since combined deletion of *c-fos* and *trp53,* leads to the spontaneous development of rhabdomyosarcomas (Fleischmann et al., 2003b). The use of tissue-specific promoters has shed light to specific roles of these proteins in cancer. Preliminary work performed in E. Wagner´s lab suggests that c-Fos has an oncogenic role, since inducible, hepatocyte-specific expression of *c-fos* leads to increased HCC development 8 months after DEN treatment. Interestingly, *c-fos* loss-of-function studies using a hepatocyte-specific cre recombinase (Alfp-cre) showed decreased tumor numbers and size 8 months after DEN treatment. These data suggest that c-Fos primarily acts as an oncogene, but depending on the tissue context, it can act as a tumor suppressor.

Contrary to c-Fos, c-Jun overexpression in transgenic mice does not result in the development of tumors, despite its oncogenic potential *in vitro* (Grigoriadis et al., 1993; Shaulian et al., 2000; Eferl et al., 1999). c-Jun is important in the development of liver tumors, as conditional inactivation of c-Jun in the liver interferes with the initiation and development of liver tumors, respectively (Eferl et al., 2003; Min et al., 2012). Additional studies have linked c-Jun to intestinal cancer in mouse models. JNK-phosphorylated c-Jun was found to interact with TCF4 to form a complex containing c-Jun, β-catenin, TCF4 and nuclear Dishevelled. These interactions seem to enhance a feed-forward loop in which the c-Jun promoter is activated by TCF4 and β-catenin, and TCF4 transcription is regulated by c-Jun (Nateri et al., 2005; Sancho et al., 2009). Most importantly, in the APC^{Min} mouse model of intestinal cancer, genetic abrogation of c-Jun N-terminal phosphorylation or gut-specific conditional *c-jun* inactivation reduced tumor number and size and prolonged lifespan (Nateri et al., 2005). c-Jun involvement in intestinal cancer probably occurs via stimulation of progenitor cell proliferation and migration (Sancho et al., 2009).

Not only do some Jun proteins promote tumorigenesis, but also they can act as tumor suppressor genes. Whereas c-Jun is oncogenic, JunB and JunD can have anti-oncogenic effects. The antioncogenic activity of JunB was confirmed *in vivo* by using

JunB-deficient mice that carry a *JunB* transgene. The transgene rescued the embryonic lethality of *JunB*-deficient fetuses (Passegue et al., 2001), but its expression was epigenetically silenced in cells of the myeloid lineage. This resulted in progressive myeloid leukaemias, with increased proliferation of granulocytic progenitor cells. JunD acts as a tumor suppressor by reducing tumor angiogenesis by protecting cells from oxidative stress (Gerald et al., 2004). Altogether, these data indicate that although similar in structure, Jun proteins can have different roles in tumorigenesis.

In contrast to c-Fos, c-Jun and Jun-B, none of the Fra proteins have been linked to cancer in mouse models.

3.3.2.1. AP-1 functions in skin tumorigenesis

AP-1 has been involved in a number of processes in the skin, including epidermal inflammation and carcinogenesis (Eferl and Wagner, 2003). Expression of a dominant-negative AP-1 transgene protects mice from UV-, chemically- and papillomavirus-induced tumor formation indicating that AP-1 activity is essential for skin tumor development (Cooper et al., 2003; Young et al., 1999; Young et al., 2002). Furthermore, in papilloma-prone mice (K5-SOS) with a concomitant keratinocyte-specific deletion of the c-*jun* gene, skin tumor development is impaired due to reduced epidermal growth factor receptor (EGFR) expression and reduced proliferation (Zenz et al., 2003). c-Fos is dispensable for mouse skin development and homeostasis (Guinea-Viniegra et al., 2012), but it is required for the development of RAS-induced malignant SCCs in the complete *c-fos* knock-out mouse (Saez et al., 1995). Moreover, tissue-specific epidermal *c-fos* deletion in mouse tumor models or pharmacological FOS/AP-1 inhibition in human SCC cell lines induced epidermal cell differentiation and skin tumor suppression by inducing p53 and subsequently TACE (TNFα converting enzyme). TACE, in turn, promotes keratinocyte differentiation by activating the Notch signaling pathway (Guinea-Viniegra et al., 2012). Additionally, c.Jun and c-Fos-phoshorylation mouse mutants show altered papilloma formation in a papilloma-prone backgroud (K5-SOS) (Behrens et al., 2000; Bakiri et al., 2011). In contrast, transgenic mice broadly expressing exogenous c-Fos are more sensitive to repeated DMBA treatment in the skin (Sakai, 1990). On the other hand, overexpression of the viral homolog of c-Fos (v-Fos) in the

spinous layer leads to long latency, wound-associated epidermal hyperplasia (Greenhalgh et al., 1995).

4. TUMOR MICROENVIRONMENT

Tumors are recognized as organs whose complexity approaches, and may even exceed, that of normal healthy tissues. Each tumor is composed by the cancer cells and by the tumor microenvironment or stroma. The tumor microenvironment is composed of fibroblasts (cancer associated fibroblasts), leukocytes, endothelial cells, soluble factors (cytokines, chemokines, matrix metalloproteases (MMPs), growth factors) and extracellular matrix (ECM) (Hanahan and Weinberg, 2011). Interactions between the cancer cells and the microenvironment can foster an adequate environment for the cancer cells to proliferate, evade cell death, induce angiogenesis and activate invasion and mestastasis (Bhowmick et al., 2004).

4.1. Extracellular Matrix (ECM)

A major component of the tumor stroma is the ECM. ECM remodeling is an active event during tumor progression. On one hand, the ECM serves as a niche for tumor cells to survive and proliferate. On the other hand, it is s a barrier that suppresses the spreading of tumor cells. Degradation of the ECM is one of the first steps in tumor invasion and metastasis (Lu et al., 2012).

The ECM is composed of a large collection of biochemically distinct components including proteins, glycoproteins, proteoglycans and polysaccharides with different physical and biochemical properties (Bourboulia and Stetler-Stevenson, 2010). The ECM is composed of basement membrane and interstitial matrix. The ECM serves as well as a biochemical mediator in signal transduction because it binds growth factors like FGFs, vascular endothelial growth factors (VEGFs) and transforming growth factor β (TGF-β) (Lu et al., 2012).

Furthermore, ECM´s biomechanical properties also change in cancer. For example, tumor stroma is stiffer than normal stroma, indeed, in the case of breast cancer, diseased tissue can be 10 times stiffer than normal breast (Lopez et al., 2011). Part of

the increase in tissue stiffness can be attributed to excess activities of Lysyl Oxidase (LOX), which crosslinks collagen fibers and other ECM components. Importantly, a study using mouse genetics has shown that overexpression of LOX increases ECM stiffness and promotes tumor cell invasion and progression (Levental et al., 2009). Thus, changes in the amount and composition of the ECM can greatly alter ECM biochemical properties, potentiate the oncogenic effects of various growth factor signaling pathways and deregulate cell behaviors during malignant transformation.

4.2. Matrix metalloproteases (MMPs)

MMPs are a family of zinc-dependent endopeptidases that play a crucial role in various physiological processes, such as tissue remodeling, organ development, inflammatory processes and cancer (Kessenbrock et al., 2010). MMPs are initially expressed as inactive zymogens and their activation depends on proteases, such as furin, plasmin or other MMPs (Bourboulia and Stetler-Stevenson, 2010).

MMPs can be secreted either by cancer cells or by cells in the stroma, such as macrophages (Kessenbrock et al., 2010). The compartmentalization of MMPs under physiological conditions often dictates their biological function. For example, high levels of active MMP-14 at the cell membrane of metastatic cancer cells play important roles in cell migration (Friedl and Wolf, 2008). Moreover, mice overexpressing MMP3, MMP7, or MMP14 form mammary tumors (Sternlicht et al., 1999). MMPs may also be critically involved in disrupting the balance between growth and anti-growth signals in the tumor microenvironment, as they potently influence the bioavailability or functionality of multiple important factors that regulate proliferation (Friedl and Wolf, 2008). Ligands for the EGFR, which are MMP targets, are potent inducers of cell proliferation and important regulators of tissue homeostasis.

Closely related to the MMPs are the so-called ADAM (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metallopreinase with thrombospoindin motifs) families of metzincing proteinases. ADAMs are also important in cancer (Edwards et al., 2008). ADAM-10 triggers the release of soluble EGF (Epidermal Growth Factor), whereas ADAM-17 is a major sheddase of TNFα, TGF-α and epiregulin. Up-regulation of EGFR signaling results in up-regulation of MMP9, which in

turn degrades E-cadherin. The cleavage of E-cadherin has an impact on cancer cell proliferation (Maretzky et al., 2005). MMP14 and MMP2 proteolytically activate TGF-β1 (Mu et al., 2002). Several MMPs have been shown to be upregulated in different types of cancer, such as MMP10, MMP1, MMP9, MMP3 and MMP14 (Liu et al., 2011; Stott-Miller et al., 2011; Kerkela et al., 2001).

In general, MMPs are not constitutively expressed in the skin, but are induced transiently in response to the abovementioned exogenous signals to trigger the proteolytic remodelling of the ECM in physiological and pathological situations, such as tissue morphogenesis, tissue repair and in tumor cells. For example, in acute murine excisional skin wounds MMP-9, MMP-3, MMP-1 and MMP-13 are strongly induced (Madlener et al., 1998).

AP-1 plays a dominant role in the transcriptional activation of the majority of MMPs that are required for cell migration and ECM degradation (Angel et al., 2001). Tissue culture as well as mouse studies have provided evidence for the importance of the AP-1 target genes MMP-1 and MMP-3 during wound healing in keratinocyte migration and wound contraction respectively (Lund et al., 1999). MMP13 is another well-known target of c-Fos and was initially identified in c-Fos induced osteosarcomas (Tuckermann et al., 2001).

4.3. Cancer Associated Fibroblasts (CAFs)

Fibroblasts synthesize many of the constituents of the ECM contributing to the formation of the basement membrane by secreting type IV collagen and laminin, and are also a source of MMPs. Fibroblasts at the site of a tumor remain constantly activated and within the tumor stroma acquire a modified phenotype. Such activated fibroblasts within the tumor stroma have been termed CAFs. These CAFs support tumorigenesis by stimulating angiogenesis, cancer cell proliferation and invasion. Several studies using mouse models have shown that cancer cells can educate surrounding CAFs to promote tumorigenesis by secreting pro-survival factors, such as IL-6 or secrected glycoprotein stanniocacin-1 (STC1) (Erez et al., 2010) (Pena et al., 2012). Interestingly, recent studies show that genetic changes in the Notch pathway in fibroblasts can have an impact on neighboring epithelial cells, thus promoting them to proliferate and give rise to tumors (Hu et al., 2012).

4.4. Inflammation and Cancer

Links between cancer and inflammation were first made in the 19th century (Balkwill and Mantovani, 2001) on the basis of observations that tumors often arise at sites of chronic inflammation and that leukocytes were present in biopsied samples from tumors (DiDonato et al., 2012). In homeostatic tissue, resident immune cells serve as sentinels to safeguard tissue and organ integrity. Following acute damage, e.g. after infection or physical trauma, resident leukocytes limit tissue damage, while engaging tissue repair programs and facilitate reepithelialization.

In cancer, immune cells play dual roles with the potential to either eliminate or promote malignancy (Balkwill and Mantovani, 2001). Premalignant tissues contain proliferating cells harboring genomic damage that typically activate critical proliferation/survival signals. In these tissues, chronic engagement of immune cells foster survival of "initiated" cells, culminating in mitotic cell expansion and development of premalignant lesions via a similar process like upon tissue injury. When these chronic inflammatory-type events are sustained, neoplastic progression can ensue (Figure 6).

4.4.1. Tumor-protective inflammation

Initially it was believed that leukocytic infiltrates in developing tumors were an attempt by the host to eradicate formation and progression of incipient neoplasias, late-stage tumors and micrometastasis (de Visser et al., 2006). The role of defective immunological monitoring of tumors seems to be validated by the striking increase in certain cancers in immuno-compromised individuals (Vajdic and van Leeuwen, 2009). However, the great majority of these are virus-induced cancers, suggesting that much of the control of this class of cancers normally depends on reducing viral burden in infected individuals, in part through eliminating virus-infected cells (Pages et al., 2010).

Patients with colon and ovarian tumors that are heavily infiltrated with Cytotoxic T Lymphocytes (CTLS) and Natural Killer (NK) cells have a better prognosis than those that lack such abundant killer lymphocytes. Indeed, extensive infiltration of NK cells in human gastric or colorectal carcinoma is associated with favorable prognosis (Ishigami et al., 2000).

In recent years, an increasing body of evidence, both from genetically engineered mice and from clinical epidemiology, suggests that the immune system operates as a significant barrier to tumor formation and progression, at least in some forms of non-virus-induced cancer. In mouse models of skin cancer, $CD4^+$ and $CD8^+$ T cells can have different roles. Kopan and colleagues have recently shown that $CD4^+$T cells are tumor-protective in skin cancers by using mouse models in which the Notch signaling pathway is impaired (Demehri et al., 2012). Moreover, Radtke and colleagues have demonstrated that $CD8^+$T responses are important in protecting from skin carcinogenesis (Di Piazza et al., 2012). Genetic elimination of $CD4^+$T lymphocytes in female HPV16 mice that undergo estrogen treatment to predispose them to cervical carcinogenesis resulted in a 10-fold increased tumor burden and a 20% increase in carcinoma incidence with estrogen-treated HPV16 controls (Daniel et al., 2005). Altogether, these data suggest a tumor-protective function of the adaptive immune responses in epithelial tumors.

4.4.2. Tumor-promoting inflammation

It is well established that inflammatory processes both hinder and facilitate cancer by fostering infiltration of leukocytes and promoting stromal remodeling (Demaria et al., 2010). Inflammatory cells are an important source of cytokines and growth factors, such as IL-6 and EGF as well as metalloproteases that foster immune cell survival and tumorigenesis (DiDonato et al., 2012).

A clear example is evident in patients with ulcerative colitis and patients with Chrohn´s disease, the two major forms of inflammatory bowel disease (IBD). There is a strong correlation between patients bearing IBD and the risk of colon cancer (Monteleone et al., 2012). In the case of pancreatic cancer (or Pancreatic Ductal Adenocarcinoma or PDAC) there is a strong correlation between inflammation in the pancreas and the risk of developing a PDAC in human and mouse models (Dite et al., 2012; Greer and Whitcomb, 2009; Grote et al., 2012; Carriere et al., 2011)

Several epidemiological studies have shown that patients with autoimmune disease, in particular, rheumatoid arthritis, Systemic Lupus Erythematosus (SLE), celiac disease and inflammation-induced pulmonary fibrosis, are predisposed to certain types of non-hematogenous malignancies, e.g., lung carcinoma, non-melanoma skin cancer and

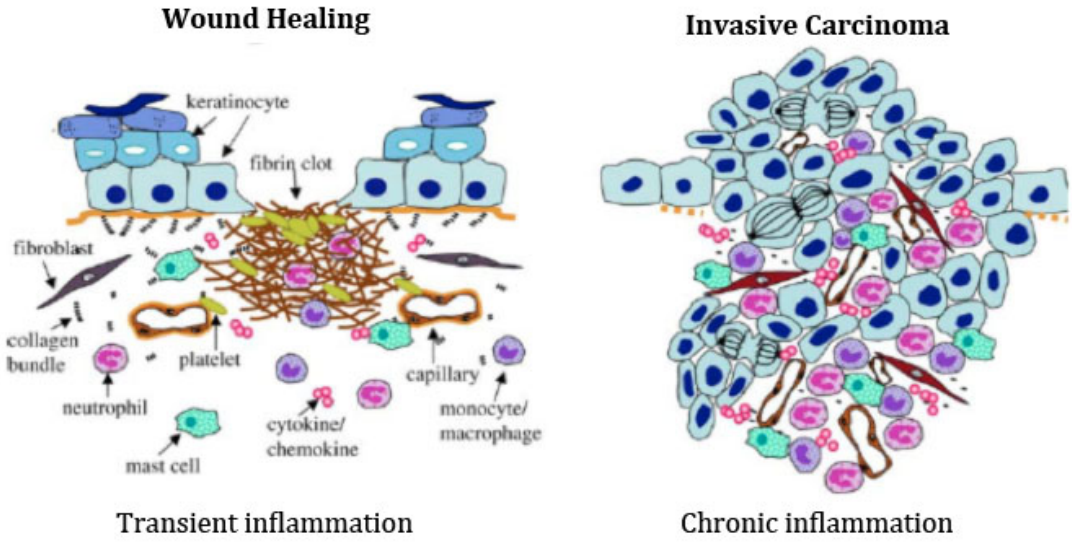
cervical atypia (Mellemkjaer et al., 1996). Overall, these data indicate inflammatory responses may be detrimental to the development of certain cancers.

4.4.3. Skin inflammation and cancer

"Cancers are wounds that never heal" Harold Dvorak. 1984.

To understand the contribution of the inflammatory microenvironment to the development and progression of epithelial skin cancer, it is important to understand how it contributes to physiological processes, such as epidermal wound healing and infection.

Figure I5.



Wound healing *versus* invasive tumor growth. Comparison between wound healing and invasive carcinoma. *Adapted from Mueller et al. 2006.*

The human adult epidermal wound healing process can be divided into 3 distinct phases: inflammatory, proliferative and remodeling (Balkwill and Mantovani, 2001). After injury a blood clot is formed and a cascade of events starts. First there is inflammation, to prevent infection and remove debris. Then, fibroblast proliferation as well as ECM remodeling, angiogenesis and finally, deposition of new connective tissue, which is known as granulation tissue. Keratinocytes are stimulated to proliferate and migrate over the granulation tissue to repair the epidermis (Arwert et al., 2012). Once

the epidermis has re-epithelized, keratinocytes and fibroblasts synthesize ECM proteins that are incorporated into the new basement membrane.

When tissue homeostasis is chronically perturbed, interactions between innate and adaptive immune cells can be disturbed. A failure to appropriately engage or disengage either arm of the immune system can result in excessive tissue remodeling, loss of tissue architecture due to tissue destruction, protein and DNA alterations due to oxidative stress and finally increase the risk of cancer development. Tumors have often been referred to as 'wounds that never heal'. Indeed, gene-expression-profiling analyses were performed to make a direct comparison between wounds and tumors, and it was found that a wound-response gene expression pattern can predict metastasis and survival likelihoods in patients with breast cancer (Chang et al., 2005)

In humans, certain inflammatory diseases, such as actinic keratosis (Berhane et al., 2002) of the skin predispose patients to develop cutaneous SCCs suggesting the inflammatory cell infiltrate is promoting skin tumor development (Gronhagen et al., 2012).

Mouse models have been important tools to study the contribution of different inflammatory cell types in the development of cutaneous SCCs. Hanahan *et al* generated a transgenic mouse model of epithelial carcinogenesis in which Human Papillomavirus type 16 was expressed from the control of the keratin 14 promoter (basal layer of the epidermis) (Arbeit et al., 1994). These mice develop by 1 month of age hyperplasias with 100% penetrance and by 1 year of age, 60% of these mice develop skin cancers, 50% of which are SCCs (Junankar et al., 2006). Using this mouse model the inflammatory cell infiltrate was characterized throughout the different stages of epithelial carcinogenesis (Junankar et al., 2006). Moreover, the contribution of different leukocytes was analyzed in the promotion of SCCs by genetically depleting different subsets of inflammatory cells (Table I1). In fact, certain populations of leukocytes are important promoters, since $CD4^{+}$T or Mast cell depletion caused a delay in the appearance of tumors in a skin SCC-prone genetic mouse model (K14-HPV16 mice, Table I1) (Daniel et al., 2003; Coussens et al., 1999).

Table I1.

Mouse cancer model	Target organ	Immune modulation	Result*
K14-HPV16	Skin	Mast-cell deficiency ($Kit^{W/Wv}$)	Decreased keratinocyte proliferation; decreased angiogenesis
K14-HPV16	Skin	$CD4^+$ T-cell deficiency	Decreased $CD11b^+$ infiltration; decreased cancer incidence
K14-HPV16	Skin	$CD8^+$ T-cell deficiency	No effect
K14-HPV16	Skin	T- and B-cell deficiency (RAG1-deficient mice)	Decreased $CD45^+$ infiltration; decreased angiogenesis; decreased keratinocyte proliferation; decreased cancer incidence
K14-HPV16 and *Mmp9*-null	Skin	Transplantation with bone marrow cells that express MMP9	Increased keratinocyte proliferation; increased angiogenesis; increased cancer incidence
K14-HPV16/E2	Cervix	$CD4^+$ T-cell deficiency	Increased cancer burden; increased cancer incidence;

Immunomodulation of skin cancer by selectively depleting different immune-cell types. *Adapted from Coussens et al.* 2011.

Carcinogens such as DMBA/TPA have also been widely employed to mimic the multifaceted steps in the development of SCCs. DMBA/TPA treatment performed in SCID mice (deficient for mature $CD4^+$ and $CD8^+$ T cells) showed a five fold decrease in the penetrance of skin tumor development. However, when used in Rag1 or Rag2 knock-out mice (also deficient in mature T cells) no difference in skin tumor penetrance was observed compared to wild-type littermates (Kemp et al., 1999)

It is known that the epidermis harbors distinct subsets of T cells, which comprise resident, interaepithelial lymphocytes (IELs) and infiltrating systemic T cells, respectively (Hayday et al., 2001). These two populations have different functions and display highly distinct gene expression profiles and T cell receptor usage. Whereas the murine and human systemic T cell compartments are dominated by $TCR\alpha\beta^+$ cells, the IEL repertoires are disproportionately enriched in $TCR\gamma\delta^+$ cells. $TCR\delta^{-/-}$ mice are highly susceptible to the development of SCCs induced by different methods (Girardi et al., 2001). However, $TCR\alpha\beta^{-/-}$ mice after DMBA and high doses of TPA are more resistant to tumor development compared to wild-type littermates (Girardi et al., 2003). Overall these data indicate that the innate immune system, is an important contributor of the development of SCC

Objectives

Little is known about the contribution of skin inflammation and the inflammatory cell component to the development of cutaneous Squamous Cell Carcinomas. The main objective of this thesis was to analyze the function of c-Fos/AP-1 transcription factor in epidermal homeostasis and carcinogenesis, and to study whether c-Fos mediates inflammatory responses in the skin that promote skin cancer development.

Therefore, the specific goals for this thesis project were:

1. Generate a mouse model to inducibly express *c-fos* in the epidermis to uncover deregulated pathways that may interfere with epidermal homeostasis, affect inflammatory processes and eventually impact on skin cancer.
2. Investigate the underlying molecular mechanisms through the identification of novel c-Fos target genes and perform functional assays to address the relevance of these genes in skin inflammation and cancer development
3. Dissect whether c-Fos and the downstream transcriptional target genes could be used as potential targets for the development of therapies to treat human skin cancer.

Objetivos

Actualmente se sabe poco acerca del papel que ejercen los procesos inflamatorios de la piel en el desarrollo de carcinomas epidermoides. Por esto, el principal objetivo de esta tesis fue analizar las funciones del factor de trascripción c-Fos/AP-1 en la homeostasis de la piel y en la carcinogénesis para entender cómo c-Fos regula las respuestas inflamatorias de la piel que pueden promover del desarrollo de carcinomas epidermoides.

Por lo tanto, los objetivos específicos de este projecto de tesis fueron:

1. Generar un modelo de ratón que exprese de manera inducible el factor de transcription *c-fos* en la epidermis para descubrir las vias de señalización que se alteran cuando los niveles de c-Fos son elevados y pueden interferir con la homeostasis epidérmica, mediar procesos inflamatorios en la piel y tener un impacto en el desarrollo de cancer de piel.
2. Investigar el papel de c-Fos mediante el descubrimiento de nuevos genes diana cuya expresión está controlada por c-Fos. Desarrollar ensayos funcionales para evaluar el papel de estos genes diana en el desarrollo de procesos inflamatorios de la piel y finalmente cancer.
3. Investigar si c-Fos y sus genes diana pueden ser dianas para el desarrollo de fármacos para tratar los carcinomas epidermoides.

Materials and Methods

1. MICE

1.1. Study approval

Mice were kept in the animal facility in accordance with institutional policies and federal guidelines. Animal experiments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain). Human tumor samples were obtained after approval from the Ethics Comittee of Instituto de Salud Carlos III (Madrid, Spain).

1.2. Generation of the tet-switchable *c-fos* allele

To generate inducible, titrable and switchable expression of *c-fos* we made use of the Tetracycline-Controlled Transcriptional Activation method. This system permits the switchable expression of a specific gene upon the presence or abscence of the tetraycline antibiotic or one of its derivatives (Doxycycline) (Gossen and Bujard, 1992).

The tetracycline (tet)-switchable *c-fos* allele was introduced 3' of the *col1a1* locus using a recombinase-mediated single-copy transgene integration strategy in Embryonic Stem cells (Beard et al., 2006). Southern blot analyses confirmed correct recombination in ES cells and correct germline transmission. Doxycycline-switchable expression of the flag tagged mouse *c-fos* cDNA sequence and the *flag* tag were confirmed in ES cells and in mice with a tetracycline activator expressed from the Rosa26 locus (Bakiri and Wagner, unpublished).

1.3. Mouse lines

1.3.1. c-Fos$^{Ep\text{-}tetON}$ and c-Fos$^{Ep\text{-}tetOFF}$ mouse lines

c-Fos$^{Ep\text{-}tetON}$ mice were generated by crossing the tet-switchable *c-fos* allele with K5-rtTA allele obtained from Sylvio Gutkind (NIH, Bethesda, USA). The K5-rtTA allele enables expression of the reverse transactivator (rtTA) under the control of Keratin 5 promoter (Vitale-Cross et al., 2004). Mice were kept in a C56BL/6 background. To generate c-Fos$^{Ep\text{-}tetOFF}$ mice the tet-switchable *c-fos* allele was crossed to the K5-tTA allele (Diamond et al., 2000). The K5-tTA allele enables expression of the transactivator (tTA) obtained from Elaine Fuchs (Rockefeller University, New York City, USA) under

the control of the K5 promoter. c-Fos$^{Ep\text{-}tetOFF}$ mice were kept in a mixed background (mix of pure C57BL/6. Double transgenic mice are referred to as c-Fos$^{Ep\text{-}tetON}$/c-Fos$^{Ep\text{-}tetOFF}$ mice or mutants. Wild-type or single transgenic littermates are always used as controls.

1.3.2. c-Fos$^{Ep\text{-}tetON}$; Rag1$^{-/-}$ mouse line

To generate c-Fos$^{Ep\text{-}tetON}$ mice lacking mature T and B cells we crossed the c-Fos$^{Ep\text{-}tetON}$ inducible mouse line to a Rag1 knock out mice, obtained from A. Ramiro (Spanish National Cardiovascular Research Centre, Spain) (Mombaerts et al., 1992).

1.4. Mouse Genotyping

Genotyping was performed by PCR analyses of genomic DNA from tail biopsies (see table M1). A single PCR reaction was prepared in a final volumen of 24 µl using 2.5 µl of Buffer (Sigma-Aldrich®) with Mg^{2+}, 2µl of 10µM primers diluted in water, 2µl of 2.5mM dNTPs (Sigma-Aldrich®), 17.5 µl of DEPC water and 0.1 µl of Taq polymerase (Sigma-Aldrich®). For the K5-rtTA PCR, 2ul of DMSO were used per reaction.

Table M1.

Allele Name	Primer	Sequence (5´-->3´)
Rosa 26-rtTA	Sense	GCGAAGAGTTTGTCCTCAACC
	Antisense #1	GGAGCGGGAGAAATGGATATG
	Antisense #2	AAAGTCGCTCTGAGTTGTTAT
Col-Fos	Sense	GCACAGCATTGCGGACATGC
	Antisense #1	CCCTCCATGTGTGACCAAGG
	Antisense #2	GCAGAAGCGCGGCCGTCTGG
K5-rtTA	Sense	CCGGATCCACCATGCCTAAGAGCCCACGACCGTCTAGAT
	Antisense	ATCTGAATGTACTTTTGCTCCATTGCGATGACT
K5-tTA	Sense	ATGCTACCATAGTCATATCGTCATGCATGCAT
	Antisense	TATCGTCATGCATGCATATGCTACCATAGTCA
Rag1	Sense	CCCGGACAAGTTTTTCATCGT
	Antisense #1	GAGGTTCCGCTACGACTCTG
	Antisense #2	TGGATGTGGAATGTGTGCGAG

1.5. Mouse treatments

1.5.1. Doxycycline treatment

Doxycycline (Sigma-Aldrich®-Aldrich®). was used to activate inducible *c-fos* expression. Initially it was supplied in drinking water in concentrations varying from 0.25-0.5g/l in the presence of sucrose (Sigma-Aldrich®). However, for most of the experiments, doxycycline was supplied in food pellets *ad libidum* (*Harlan* laboratories).

1.5.2. Chemical carcinogenesis

For DMBA/TPA-induced skin carcinogenesis, cohorts of c-Fos$^{Ep\text{-}tetON}$, c-Fos$^{Ep\text{-}tetOFF}$, at 6-8 weeks of age were shaved and 2-3 later received a single application of 100 µL of an acetone solution containing 0.5% DMBA (7,12-dimethylbenz[a]anthracene, Sigma) applied to the dorsal surface. 10^{-4}M TPA in acetone was applied twice a week to the dorsal surface. This treatment was combined (or not) with doxycycline and with Sulindac (Sigma-Aldrich®).

1.5.3. Anti-inflammatory treatment

COX1/COX2 inhibitor *Sulindac* (Sigma-Aldrich®) was supplied in drinking water in a concentration of 1.8mg/l diluted in autoclaved water. This treatment was combined with Doxycycline in the food pellets and with 0.5% DMBA applied topically only once.

1.5.4. MMP inhibitory treatment

6-8 week old control and c-Fos$^{Ep\text{-}tetON}$ mice were treated with Dox for four weeks. TAPI-1 (MMP10 inhibitor, Peptides International) was injected intraperitoneally (IP) every other day three times a week during 4 weeks. TAPI was diluted in 5%DMSO in saline. The final concentration used was 10mg/kg.

1.6. Skin barrier assays

1.6.1.Transepidermal water loss (TEWL) in vivo epidermal barrier assay

Measurements in pups at P1 were performed with Tewameter TM300 (EnviroDerm Services) following the manufacturer´s instructions. Units are given in gram per hour per square meter.

1.6.2 Toluidine Blue in vivo epidermal barrier assay

This assay modifies skin to permit barrier-dependent penetration by histological dyes. Euthanized newborn pups were rinsed in PBS (1X) and immersed in 25%, 50%, 75% and 100% methanol for 1 minute each successively; pups were then rehydrated in PBS and stained in 0.1% toluidine blue for 10 minutes at room temperature. They were washed briefly in PBS (1X) and photographed immediately.

2. HISTOLOGICAL ANALYSIS

2.1. Fresh frozen tissue

Tissues were embedded in optimal cutting temperature (OCT) compound (Sakkura Finetek, Torrance, CA), snap-frozen in dry ice, and 6µm cryostat sections were cut. Immunofluorescence analyses were performed as described previously (Perez-Moreno et al., 2006), using the following antibodies: s100a7a15 (home-made, kindly provided by Ronald Wolf, Ludwig-Maximilian University, Munich,) and Alexa488- or Alexa594-coupled secondary antibodies (Invitrogen™). DAPI (Invitrogen™).

2.2. Formalin-fixed paraffin-embedded tissue

Tissues were fixed in 10 % formalin, embedded in paraffin, 4µm sectioned and dried at 37ºC overnight for further processing. Immunohistochemical detection was performed using antibodies against different epitopes: c-Fos (sc-52, Santa Cruz Biotechnology Inc.), Flag (Cell Signaling), CD3 (Santa Cruz Biotechnology Inc.), Ki-67, MMP10 (DAKO), p-Stat3 (Cell Signaling), Keratin 1, 5, 6, 10, 14, Filaggrin, Loricrin (Covance), human CD4 (IF6 clone, Novocastra). Sections were subjected to antigen retrieval using citrate buffer pH6 (1mM sodium citrate, 0.05%Tween 20) and cooked in a pressure cooker. Immunohistological stainings were performed according to the manufacturer´s protocol (either the EnVision DAB+ kit (DAKO) for rabbit IgG or the VECTASTAIN Elite ABC Kit (Vector Labs) for rat, goat or mouse IgG).

2.3. Human samples and Tissue Microarray (TMA) preparation

Human skin SCC samples obtained after informed consent (ethics permission number is 125/10/2012 EK 405/2006) and approval were provided and evaluated by Dr. Peter Petzelbauer (Medical University of Vienna, Austria).

Production of tissue arrays from paraffin blocks from BCC and SCC lesions (from the dermato-pathological data bank) according to ethics committee permission 405/2006 and extension 125/10/2012). In brief, by this technique 0.5 mm punches are taken out of the paraffin-embedded material by parallel viewing of H&E-stained sections.

2.4. Nile Red staining

Nile red (Sigma-Aldrich®) staining was performed on frozen sections for 2 min with 0.15mg/ml Nile Red in 75% glycerol. Stock solution of nile red at 0.5 mg/ml was prepared in acetone. To prepare the working solution, 0.05ml of stock solution is added to 50ml of a 75:25 glycerol-water mixture. To stain the sections, a drop of working solution was added on the frozen tissue and it was covered with a coverslip. The section was analyzed in a Nikon fluorescence microscope.

3. PROTEIN ANALYSES

3.1. Protein extraction and quantification

Protein isolation for Western blot was performed in RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) containing a protease inhibitor cocktail (Sigma-Aldrich®), 0,1mM Na_3VO_4, 40mM β-glycerophospate, 40mM NaPPi, 1mM NaF and tissues were homogenized using Precellys 24 (Bertin technologies). For protein lysates from cultured keratinocytes, cells were scraped with 100ul (for each well in a 6 well plate) with a cell scraper and kept in ice for 15 minutes. The cell lysate was spun down at maximum speed and supernatant was collected. Protein lysates were quantified by using Pierce BCA Protein Assay Reagent (Thermo Scientific).

3.2. Immunoblotting

Equal amounts of protein (20-50ug) were separated by SDS-PAGE on 4-12 Western blot analysis was performed according to standard procedures using the following antibodies: c-Fos (BD-Pharmingen), Flag (Sigma-Aldrich®), Vinculin (Sigma-Aldrich®), MMP10 (DAKO), S100a7a15 (provided by Dr. Ronald Wolf), β-Actin (Sigma-Aldrich®), GAPDH (Sigma-Aldrich®).

The blots were incubated with the appropriate secondary horseradish peroxidase-coupled antibodies (GE Healthcare) and developed using the Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare) and Amersham ECL Hyperfilms (GE Healthcare). Alternatively, blots were incubated with Alexa-Fluor 680 coupled secondary antibodies (Invitrogen™) and visualized using the Odyssey Imaging system (Li-Cor).

3.3. Chromatin Immunoprecipitation (ChIP)

ChIP analysis was performed with a ChIP Assay Kit (Upstate) according to the manufacturer's instructions. Antibodies used were c-FOS (Santa Cruz Biotechnology Inc.). Cells were plated as for keratinocyte culture in order to exclude any other cell type in the sample. Isotype antibodies were used as controls (BD Biosciences). Bound and unbound fragments were run in agarose gels or quantified by real-time PCR when indicated.

4. RNA ANALYSES

4.1. RNA extraction from tissues or cells

Total RNA was isolated using Trizol (Sigma-Aldrich®) according to the manufacturarer´s instructions. Complementary DNA was synthesized using 1μg of RNA, Ready-To-Go-You-Prime-First-Strand Beads (GE Healthcare) and random primers were used (Sigma-Aldrich®) as described in the manufacturer´s protocol. Quantitative reverse transcriptase PCR reactions were performed using GoTaq® RT-qPCR Master Mix (Promega) and Eppendorf fluorescence thermocyclers (Eppenddorf). The $2^{-\Delta\Delta CT}$ method was used to quantify amplified fragments. Expression levels were normalized

using at least two housekeeping genes (*gapdh*, *hprt*). Primer sequences are shown in Table M2, and M3.

4.2. RNA extraction from FACS-sorted cells

This protocol was used to extract RNA from a very low number of cells (down to 10.000 cells). Cells were collected in either FACS tube (when using the BD Influx Sorter) or 1.5ml eppendorf tubes (when using the BD FACSAria III). Cells were spun down and resuspended in 350µl of RLT buffer (Qiagen) + 3.5 µl of B-Mercaptoethanol (Sigma-Aldrich®). The cells were disrupted with an insulin syringe 10 times up and down. A 150µl of chloroform was added and the whole suspension was carefully mixed and spun down at 13000rpm for 10 min at 4C. Carefully, the upper layer phase was transfered to a clean 1.5ml Eppendorf tube. 400 µl of isopropanol with 4µl of Ambion® linear acrylamide (Invitrogen™) was added on top. This mix was left to precipitate overnight at -20C. Next morning, the mix was spun down 30 minutes at 13200rpm at 4C. The pellet was washed twice with 70% EtOH in DEPC water. The mix was spun down and the pellets were left to air dry for a couple minutes. Finally, pellets were resuspended in 15ul of Rnase-free water.

Complementary DNA was synthesized using 100ng of RNA and following the instructions of the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™).

Table M2.

Allele Name	Primer	Sequence (5´-->3´)
c-fos	Sense	CTGTCACCGTGGGGATAAAG
	Antisense	CCTACTACCATTCCCCAGCC
c-Fos-flag	Sense	ATGGGGCTCTCCTGTCAACACAC
	Antisense	TGTCGTCGTCGTCCTTGTAG
mmp10	Sense	TATGTGTGTCACCGTCCTGG
	Antisense	AGCCACAAGTTGATGCTGTG
s100a7a15	Sense	TGA AGG GTC CAT CAG TCA
	Antisense	CTA GTA GAG GCT GTG CT
hprt	Sense	ATCGTCTACTGCTATCTGCGT
	Antisense	GCTATCTGCGTATCGTCTACT
gapdh	Sense	TTGATGGCAACAATCTCCAC
	Antisense	CGTCCCGTAGACAAAATGGT
notch-1	Sense	TCTTACACGGTGTGCTGAGG
	Antisense	GAATGGAGGTAGGTGCGAAG
	Sense	GTCACCTCGTTCATGCACTC

hes-1	Sense	GTCACCTCGTTCATGCACTC
	Antisense	TCTGGAAATGACTGTGAAGCA
keratin 5	Sense	CAGTGTGCCAACCTCCAGAACG
	Antisense	AGCCCGCTACCCAAACCAAGAC
keratin 14	Sense	GACGCCGCCCCTGGTGTG
	Antisense	GGTGGCGATCTCCTGCTC
keratin 1	Sense	CTCTACGTAGTTACCACAACAAT
	Antisense	CAATCCTCTACGTAGACGTA
keratin 10	Sense	GGAGGGTAAAATCAAGGAGTGGTA
	Antisense	TCAATCTGCAGCAGCACGTT
filaggrin	Sense	CTAGAGGGCATGAGTGTAGTCA
	Antisense	CAAGACTGGACAGTTGGCTGG
loricrin	Sense	TCACTCATCTTCCCTGGTGCTT
	Antisense	GTCTTTCCACAACCCACAGGA
ifn-γ	Sense	GAGCTCATTGAATGCTTGGC
	Antisense	GCGTCATTGAATCACACCTG
il-4	Sense	ACCTTGACGGTGTTCATACAGT
	Antisense	CTGCTCCTATTCGACCACTATCT
il-5	Sense	CCCAGGGCGACTGTAACATC
	Antisense	GCAATGTAGATCCTCATGGCAT

Table M3.

Allele Name	Primer	Sequence (5´-->3´)
C-FOS	Sense	GTGACCGTGGGAATGAAGTT
	Antisense	CCGGGGATAGCCTCTCTTAC
MMP10	Sense	CTGATGGCCCAGAACTCATT
	Antisense	ACTGGAACCCTGAACCTGAA
S100A7	Sense	CAGGCTTGGCTTGTCAATCT
	Antisense	TCATCCTTCTACTCGTGACGC
HPRT	Sense	CCTGGCGTCGTGATTAGTGAT
	Antisense	AGACGTTCAGTCCTGTCCATAA

4.3. Genome-wide expression analyses

RNA of samples was hybridized to the Illumina MouseRef-8 V2 R3 BeadChip array according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). Microarray scanning was done using a Beadstation array scanner. Data preprocessing and quality control was conducted using packages of the Bioconductor project implemented at DKFZ in-house-developed ChipYard framework for microarray data analysis (http://www.dkfz.de/genetics/ChipYard/). In summary, microarray probes

were annotated based on Ensembl (version 58) using an in house BLAST-based pipeline. Before normalization with variance-stabilizing transformation (VST) and robust spline normalization (RSN) algorithms, beads with signals below the negative controls were removed and positive negative and hybridization controls were excluded.

Normalized log2 transformed microarray data were used to calculate the gene fold change value (i.e. how many times the intensity of gene expression changed compared to the control). FC value has no measure of statistical significance, but is biologically relevant and specifically usable for data, which other than the time series has no other replication. Genes were ranked according to the Euclidian distance between the maximum expression within a probe set and the mean expression of a probe set over all time points (as show in (Busch et al., 2008)), both scaled (normalized) to the maximum value measured in each dataset.

5. FLOW CYTOMETRY

A similar protocol was used to perform Flow cytometry analyses and Cell Sorting. Samples were taken from back skin of c-Fos$^{Ep\text{-}tetON}$ mice or controls treated with doxycycline for 2 and 4 weeks. Different protocols were used to homogenize back skin and lymph nodes.

5.1. Back skin-specific protocol

Back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice was shaved and removed from the mouse. The fat layer was eliminated using a scalpel. The skin was cut in small pieces with scissors and transfered to MACS tubes (Miltenyi) in 4ml of plain RPMI (Gibco®) containing 120ul of DNase (Sigma-Aldrich®) at 4mg/ml diluted in RPMI. 200µl of liberase was as well added (Roche) at 5mg/ml. The skin was incubated for 30 minutes in a water bath at 37C. Homogenization was performed using the program B01 in a MACS homogenizer twice. To quench the enzymatic reaction, 10ml of 10% chelated FBS-RPMI was added to the homogenate. The mix was filtered through a 70µm cell strainer and then through a 40um cell strainer. Each filter was rinsed with 5ml of PB low-BSA buffer (2mM EDTA, 0.5% BSA, PBS) to collect the cells remaining in the strainer. After, the cell

preparation was spun down 10 minutes at 300g at room temperature. The supernatant was removed and the pellet was resuspended in 1 ml of red blood cell lysing (Sigma-Aldrich®) buffer. The cells in the RBC lysing buffer were incubated for 5 min the fridge. 5ml of PB buffer was added and the whole mix was spun down at 300g for 5 minutes at 4C. The pellet was resuspended in 300μl of PB high BSA buffer (2mM EDTA, 2% BSA, PBS). Fc block (BD Pharmingen) was added in a dilution of 1/200 and kept 30 minutes on ice. 30 minutes after, the mix was divided into different conditions (depending on the number of the different populations tested).

5.2. Lymph node-specific protocol

Skin draining lymph nodes were removed from the mouse and kept in 5%FBS RPMI. Lymph nodes were smashed with a plunger against a 40μm cell strainer. The strainer was washed with 5%FBS RPMI. Cells were spun down at 300g for 10 minutes. Finally, leukocytes were resuspended in PB buffer low-BSA and staining was carried out.

5.3. Flow cytometry analyses

For flow cytometry analyses, stainings were performed using different combinations of the following antibodies depending on the experiment.: NK1.1 PerCP Cy5.5 (1/200) (BD Pharmingen), CD25 APC 1/200 (BD Pharmingen), CD45.2 FITC 1/400 (BD Pharmingen), γ/δ TCR Brilliant Violet 421 1/200 (Biolegend), CD3 (BD Pharmingen), CD4 PECy7 1/100 (BD Pharmingen), CD8 (BD Pharmingen), Gr1 FITC (BD Pharmingen), CD45 1/500 (BD Pharmingen), CD11b PErCPCy5.5 1/200 (BD Pharmingen), B220 PE 1/200 (BD Pharmingen), CD11c PE 1/100c (BD Pharmingen), CD69 PE (BD Pharmingen), CD44 APC 1/200 (BD Pharmingen). At the same time the FMOs (Fluoresence Minus One) controls were prepared. In these controls you add every single antibody in the antibody cocktail used minus one. The antibodies were incubated with the cells for 30 minutes in ice protected from light. After staining, two washes were performed with PB buffer. Depending on the group of fluorophores used, we employed either DAPI (5ul of a stock at 2mg/ml) or Acqua live (1/1000 dilution from the original stock in PB buffer). Samples were incubated for 30 minutes at room temperature and afterwards washed twice with PB buffer. Cells were fixed with 1%PFA in PB buffer in a final volume of 400μl for 30 min in the fridge. Finally, cells were washed and next

morning samples were analyzed in different Cell Analyzers (BD FACSCanto™, BD LSRII Fortessa™ or BD FACSCalibur™). Beads from BD™ CompBeads were used to compensate every single color used in each experiment. For this purpose, positive beads were incubated separately with each antibody, then washed and finally the negative beads were added. Analyses were performed using FlowJo software.

5.4. FACS sorting

Cell suspensions were stained with CD3 APC 1/200 (BD Pharmingen), CD4 PECy7 1/100 (BD Pharmingen), and Gr1 FITC (BD Pharmingen) for 30 minutes in ice. Cells were washed with PB buffer twice and right before sorting, 5ul of DAPI at 2mg/ml was added. Cells were usually sorted in a BD Influx Sorter and sometimes in a BD FACSAria III.

6. CELL CULTURE

6.1. Keratinocyte primary cultures

6.1.1.Primary keratinocytes

Isolation and culture of primary keratinocytes were performed as described (Zenz et al., 2003). Briefly, the epidermis was separated from the dermis by incubation in 0.75% trypsin (Gibco®) at 37°C. 60 min later, the epidermis was incubated in medium containing 250μg/ml DNase (Sigma-Aldrich®) for 30 min in a water bath. The cell suspension was filtered through a 70μm cell strainer. Keratinocytes were spun down and plated in coated dishes (Cascade Biologics™) in complete MEM (8% chelated fetal calf serum (FCS), Gentamycin, Penicyllin, Streptomycin, 0.05mM Ca^{2+} in Eagle MEM (Sigma-Aldrich®). Next day the medium was changed to serum-free keratinocyte medium (KC-SFM) (Gibco®) and c-$Fos^{fl/fl}$ were infected with adenoviruses (Ad-Cre or Adeno-green fluorescent protein (Ad-GFP) purchased from the University of Iowa with 300 particles per cell. Cells were collected at different time-points post-infection. c-$Fos^{Ep\text{-}tetON}$ keratinocytes were cultured in KC-SFM ± 1μg/ml Dox treatment starting one day after plating the cells. Keratinocytes were collected at different time points. TPA treatment was used at a concentration of 10ng/ml for 3, 5, 6, 8, 12 hours depending on

the experiment. For Calcium treatment, Ca^{2+} was diluted at a concentration of 1.8mM in KC-SFM. Keratinocytes were always grown at 32C.

6.1.3. E Low Calcium medium

Keratinocytes used for colony formation assays were kept in E-Low Calcium medium. This media was prepared as previously described (Nowak and Fuchs, 2009)

6.1.4. SCC cell lines

Epidermal-derived SCCO9 cells were kindly provided by M. Sibilia (Institute for Cancer Research, Vienna, Austria); SCCO11, SCCO22 were kindly provided by G. P. Dotto (University of Lausanne, Lausanne, Switzerland); These cell lines were cultured in DMEM 10%FBS.

6.1.5. Feeders

3T3 fibroblasts or feeders were maintained in 10% FBS DMEM F:12 (3:1) (Gibco®) with sodium bicarbonate, L-glutamine and Pen-Strep. These cells were never passaged for more than 20 passages. When feeders were ready to be used they were treated with 10ug/ml mitomycin C (stock 0.5mg/ml, dissolved in H2O) in complete medium for 2 hours at 37C, then trypsinized and replated 1/3 of their saturation density along with keratinocytes

6.4. *In vitro* proliferation assays

6.4.1. Cell Counts

$5x10^5$ c-Fos^{tetON} keratinocytes were plated in triplicate on six-well plates coated with Coating Matrix and grown in keratinocyte serum-free media. Dox treatment started one day after for 1, 2, 3, 4, and 5 days. Cells were trypsinized and counted with a neubauer chamber at indicated times.

6.4.2. Colony formation assays

To analyze colony formation properties, 1500 cells were plated in triplicate on six-well plates coated with coating matrix on top of feeders. Cells were kept in E-low calcium medium in the presence or absence of 1μg/ml of Dox. 17 days after having

plated the keratinocytes, the culture media was removed and remaining fibroblasts were washed with PBS1x until they were detached. Keratinocyte colonies were fixed in 25% glutaraldehyde in PBS 1X for 15min at RT. Then, glutaraldehyde was removed and cells were stained with 0.05% Crystal Violet in distilled Water. The dye was kept for 30min at room temperature. Colonies were washed twice with water and left them inverted to dry. The plates were scanned the colonies were quantified using Axio software. Colony number and size was measured.

6.4.3. EdU incoroporation assay

c-Fos^{tetON} keratinocytes were plated in triplicates according to the conditions cited in section 6.1. Cells were cultured in KC-SFM for different times ± 1μg/ml of Dox in the media. EdU (Click-It-EdU, Invitrogen™) was added to cell culture media at a concentration of 10μM and 2 hours later cells were carefully trypsinized, scraped from the plate and transferred FACS tubes. Keratinocytes were spun down at 500g for 15 min at 4C and the cell pellet was fixed with 100 μl of 4% PFA 15 min at room temperature. Cells were washed and resuspended in 1% BSA/PBSA. EdU incorporation was measured by Flow Cytometry analyses in a BD LSRII Fortessa™. %EdU positive cells were quantified using FlowJo software.

Statistical analyses

Unless stated differently, all experiments were performed 3 times. Data in bar graphs represent mean ± S.D. of triplicates. Statistical analysis was performed using non-directional two-tailed Student's test or one-way ANOVA tests depending on the number of groups compared. *$p < 0.05$ is considered as significant.

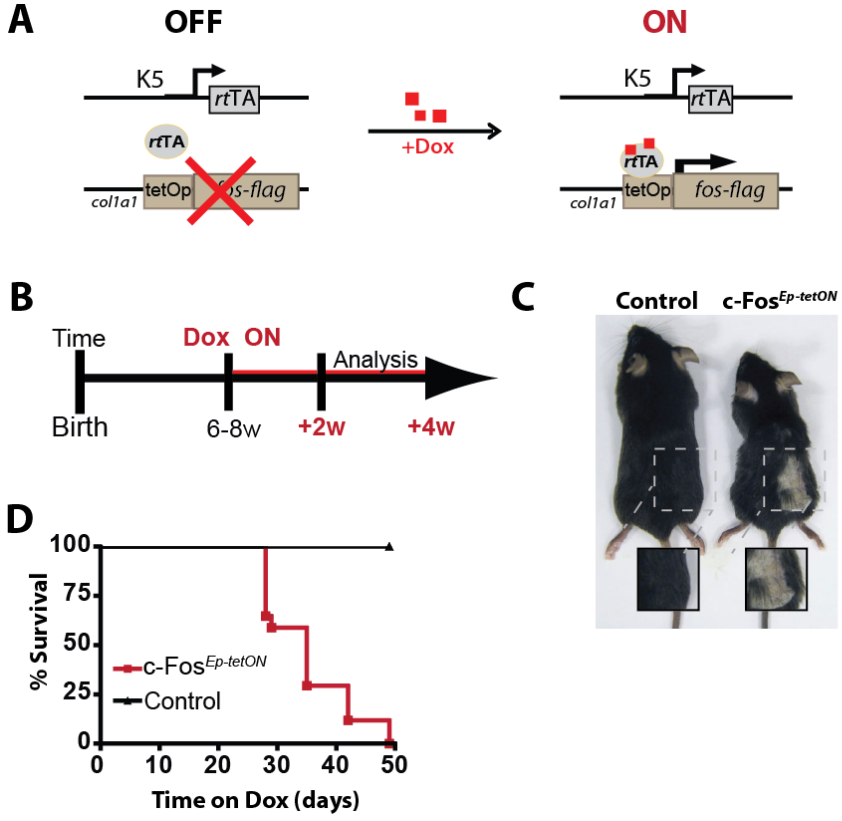
Results

1. INDUCIBLE EPIDERMAL *C-FOS* EXPRESSION IN ADULT MICE LEADS TO EPIDERMAL HYPERPLASIA WITH INCREASED PROLIFERATION

1.1. Inducible keratinocyte-specific *c-fos* expression: c-Fos$^{Ep\text{-}tetON}$ mouse model

Increased c-Fos levels have been found in SCC tumors arising in different stratified squamous epithelia such as the epidermis (Guinea-Viniegra et al., 2012; Sachdev et al., 2008). To address the question whether keratinocyte-specific c-Fos expression alters epidermal homeostasis and is sufficient to promote SCC development in the skin, we have generated a doxycycline (Dox) inducible mouse model. To achieve inducible expression of *c-fos* in keratinocytes *in vivo*, we crossed K5-rtTA mice to col$^{TetO\text{-}Fos}$ mice (Fig 1A). Double transgenic mice will be referred to as c-Fos$^{Ep\text{-}tetON}$ mice or mutants, while wild-type or single transgenic littermates will be used as controls.

Figure 1.



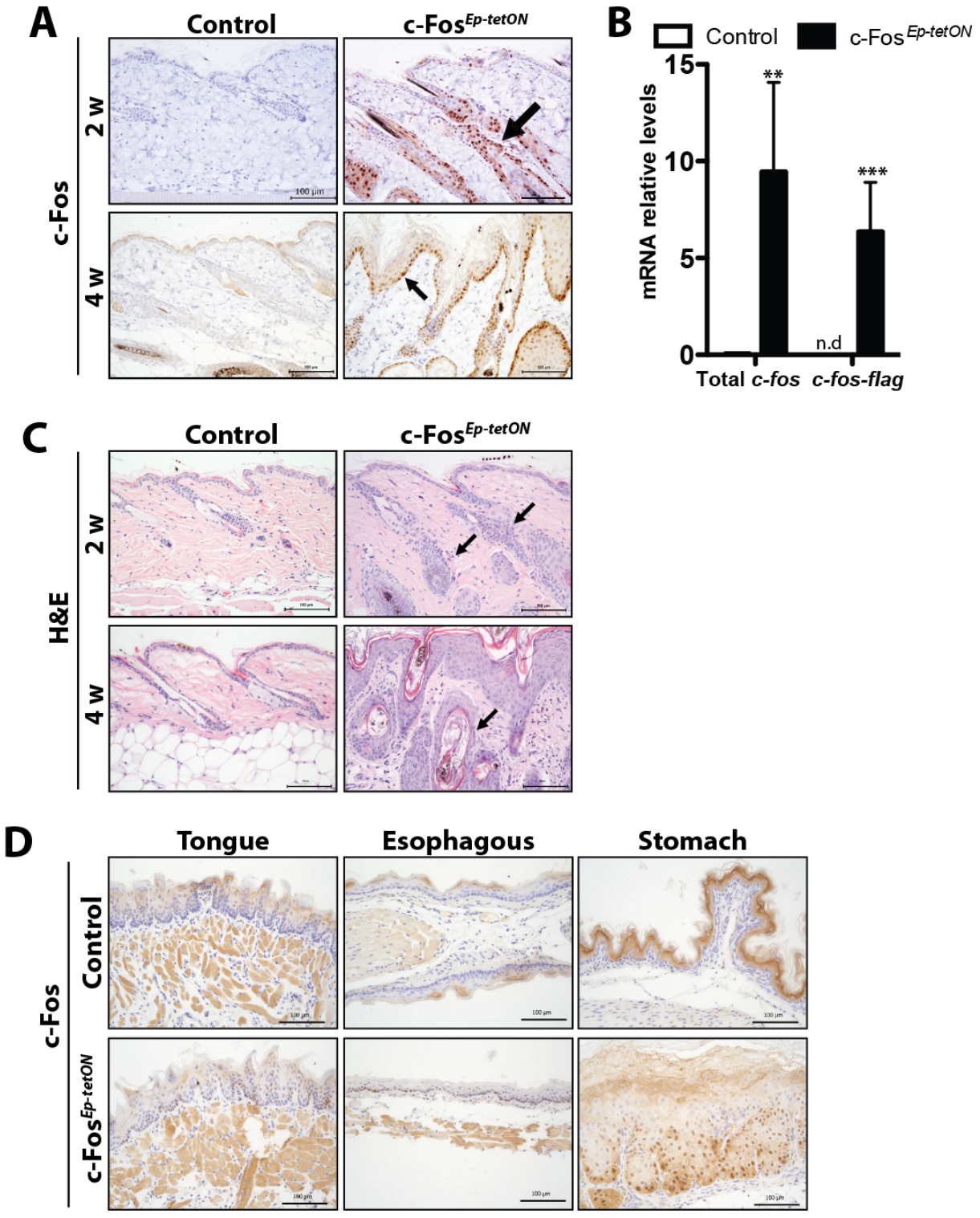
(A) Schematic representation of the c-Fos$^{Ep\text{-}tetON}$ mouse model. rtTA (reverse transactivator), tetOp (tetracycline-regulatable operator). Dox is supplied either in the drinking water (0.5mg/l) or in the food. (B) Experimental set-up. (C) Representative picture of a control and a c-Fos$^{Ep\text{-}tetON}$ mouse model treated with Dox in the food for 4 weeks. Square shows a higher magnification picture of the back skin of the mice showing the macroscopic lesions. (n= 8 controls, 8 mutants). (D) Kaplan-Meier survival curve depicting control and c-Fos$^{Ep\text{-}tetON}$ mice (n=20/17).

c-fos expression was always induced in adult c-Fos$^{Ep\text{-}tetON}$ mice from 6 to 8 weeks of age, by supplying them with Dox (Fig. 1B). c-Fos$^{Ep\text{-}tetON}$ adult mice expressing c-Fos following Dox treatment for 4 weeks showed reduced size and dramatic hair loss compared to control mice. Interestingly, the skin of these mice was thickened with cornified epidermis appearing as dry, scaly lesions (Fig.1C). About 50% of the mutant mice eventually had to be euthanized due to ethical reasons short after 4 weeks of Dox treatment (Fig. 1D)

Inducible expression of *c-fos* was detectable in the epidermis as early as 2 weeks after Dox induction by immunohistochemistry (IHC) and RT-qPCR (Fig. 2A and 2B). At 2 weeks expression was mainly localized to the hair follicles, while at 4 weeks it was clearly visible in the basal layer of the epidermis and outer root sheath of the hair follicles (Fig. 2A, arrows). Histological analyses of back skin revealed a complete disruption of the skin architecture accompanied by massive epidermal hyperplasia, hyper- and parakeratosis and cell and nuclear atypia in c-Fos$^{Ep\text{-}tetON}$ mice following Dox treatment for 4 weeks. Strikingly, already at 2 weeks after inducible *c-fos* expression, hair follicles were enlarged similar to the hair follicles in anagen or growing phase of the hair cycle compared to controls (Fig. 2C, arrows). At 4 weeks, most hair follicles were absent and foci of central keratinization within concentric layers of abnormal squamous cells were often observed (Fig. 2C). These cysts expressed early and late interfollicular epidermal differentiation markers, such as Keratin 1 (KI), Keratin 10 (K10), Loricrin and Filaggrin (data not shown), suggesting an alteration of the differentiation program of the hair follicle upon inducible *c-fos* expression.

Other keratin 5 (K5)-expressing epithelial tissues, such as the tongue and the forestomach expressed exogenous *c-Fos*, while tissues that do not express K5, such as spleen or liver, did not (Fig. 2D and data not shown). Altogether, these data indicate that inducible expression of *c-fos* in the epidermis alters epidermal homeostasis consequently disrupting the skin architecture.

Figure 2.

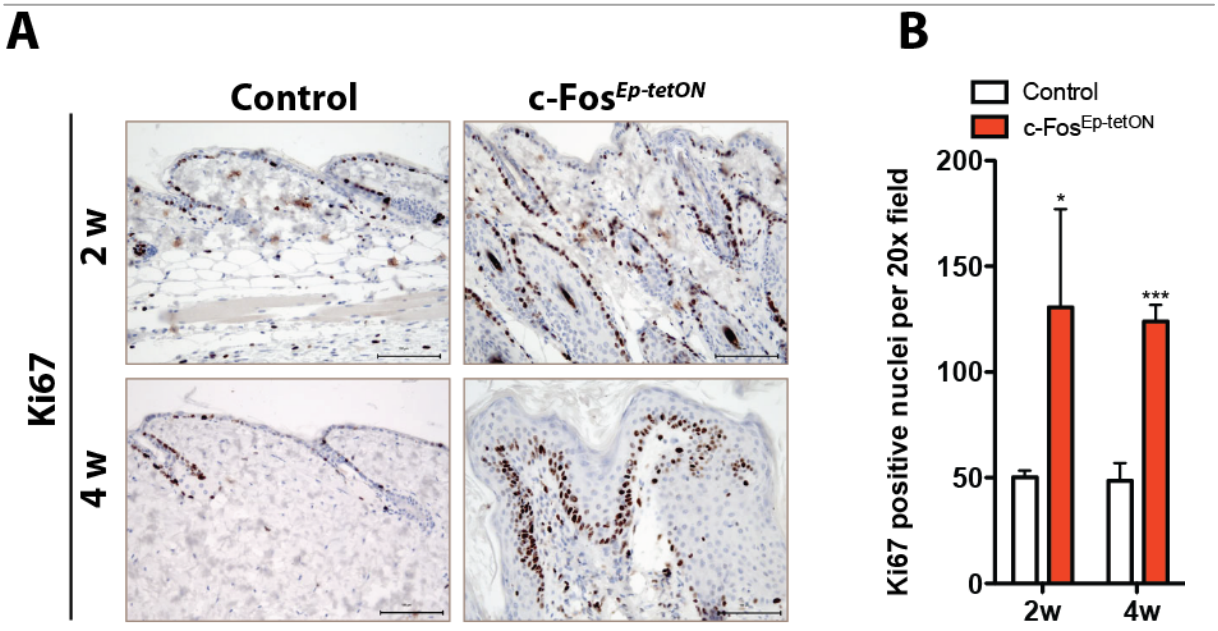


(A) Histological analyses of c-Fos in back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice after 2 and 4 weeks on Dox. (B) RT-qPCR analyses of total *c-fos* and exogenous *c-fos* (*c-fos-flag)* levels. (n=4/4/4/4). (C) H&E histological analyses of back skin of c-Fos$^{Ep\text{-}tetON}$ mice after 2 and 4 weeks on Dox. Arrow indicates hair follicle. (n=4/4/4/4). (D) Histological analyses of c-Fos in stomach, tongue and esophagous of c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks on Dox. (n=3/3).

1.1.2. c-Fos promotes proliferation *in vivo* in a non-cell autonomous manner

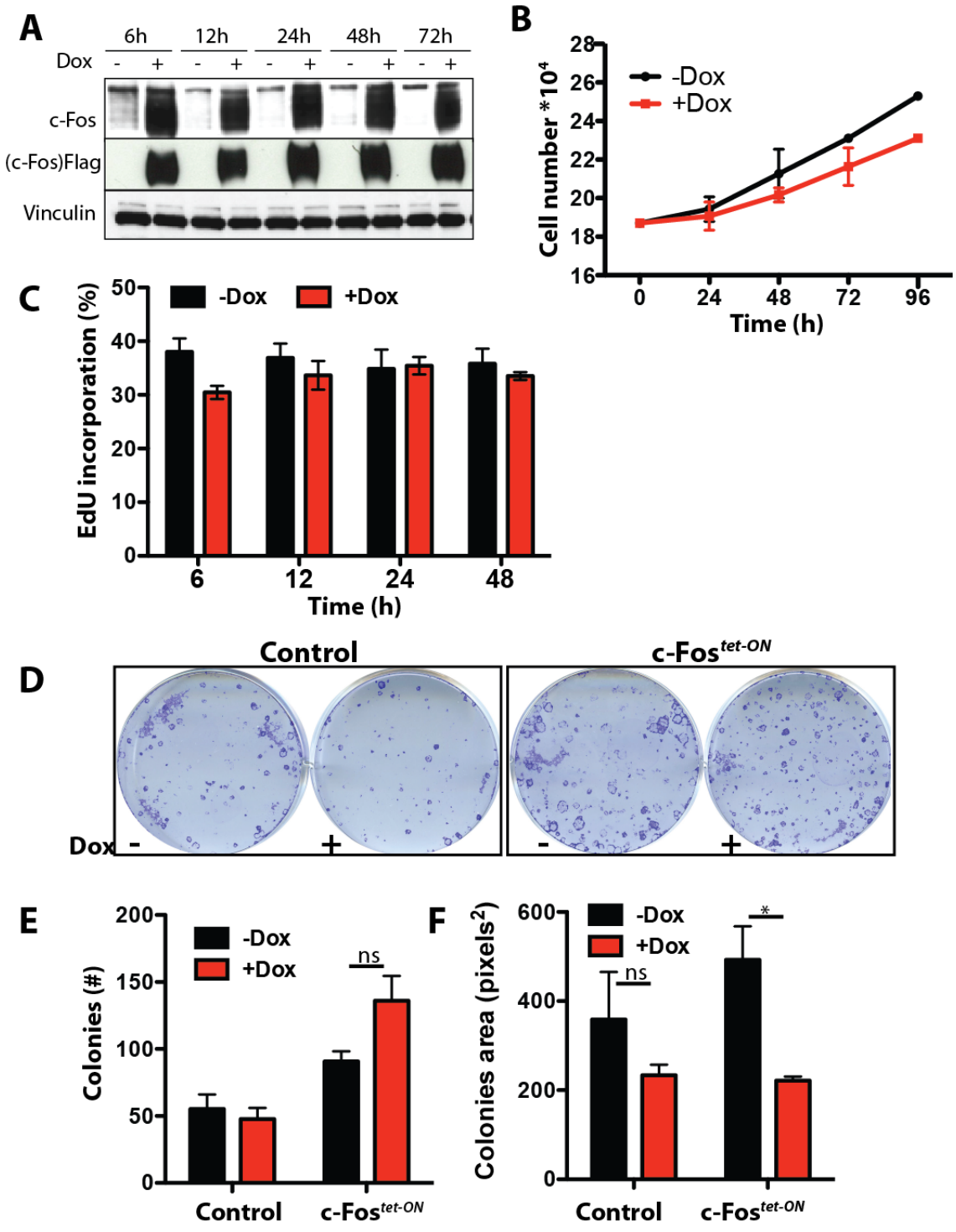
The balance between keratinocyte proliferation and differentiation must be tightly controlled to maintain epidermal homeostasis. A disruption in any of these two physiological processes leads to development of diseased skin. Therefore, we assessed proliferation *in vivo* by measuring Ki67 protein levels in c-Fos$^{Ep\text{-}tetON}$ back skin samples. Interstingly Ki67 IHC staining revealed increased proliferating cells in the basal layer of the epidermis upon *c-fos* expression as early as 2 weeks of Dox treatment (Fig. 3A and B). To evaluate whether c-Fos would promote keratinocyte proliferation in a cell-autonomous manner, we measured keratinocyte proliferation *in vitro* by using primary c-Fos^{tetON} keratinocytes from the tail. Upon Dox treatment, inducible expression of *c-fos in vitro* was detected by immunoblot analyses (Fig. 4A). However, c-Fos did not promote proliferation, as measured by keratinocyte number in culture (Fig. 4B), EdU incorporation (Fig. 4C) and colony formation assays (Fig. 4D, 4E and 4F). Altogether, these data indicate that expression of *c-fos* in keratinocytes is not sufficient to promote proliferation in a cell autonomous manner.

Figure 3.



(A) Histological analyses of c-Fos$^{Ep\text{-}tetON}$ back skin of Ki67 at indicated times on Dox (w=weeks). (B) Quantification of Ki67 positive nuclei per 20x field. 2w/4w: 2 and 4 weeks of Dox treatment. *p<0.05, ***p<0.0005.

Figure 4.



(A) Immunoblot depicting protein levels of total c-Fos total, exogenous c-Fos and vinculin in c-Fos^{tetON} keratinocytes at 6, 12, 24, 48 and 72 hours ±Dox. (B) c-Fos^{tetON} keratinocyte number quantification in cultured ±Dox for 0, 24, 48, 72 and 96h. (C) EdU incorporation assay in c-Fos^{tetON} keratinocyte treated ±Dox for 6, 12, 24 and 48 hours. (D) Picture of colonies formed from control and c-Fos^{tetON} keratinocytes treated ±Dox in a colony formation assay. Colonies are stained with crystal violet. (E) Quantification of the number of colonies. (F) Quantification of the colony size (by measuring the number of square pixels of each colony). ns= Not significant.

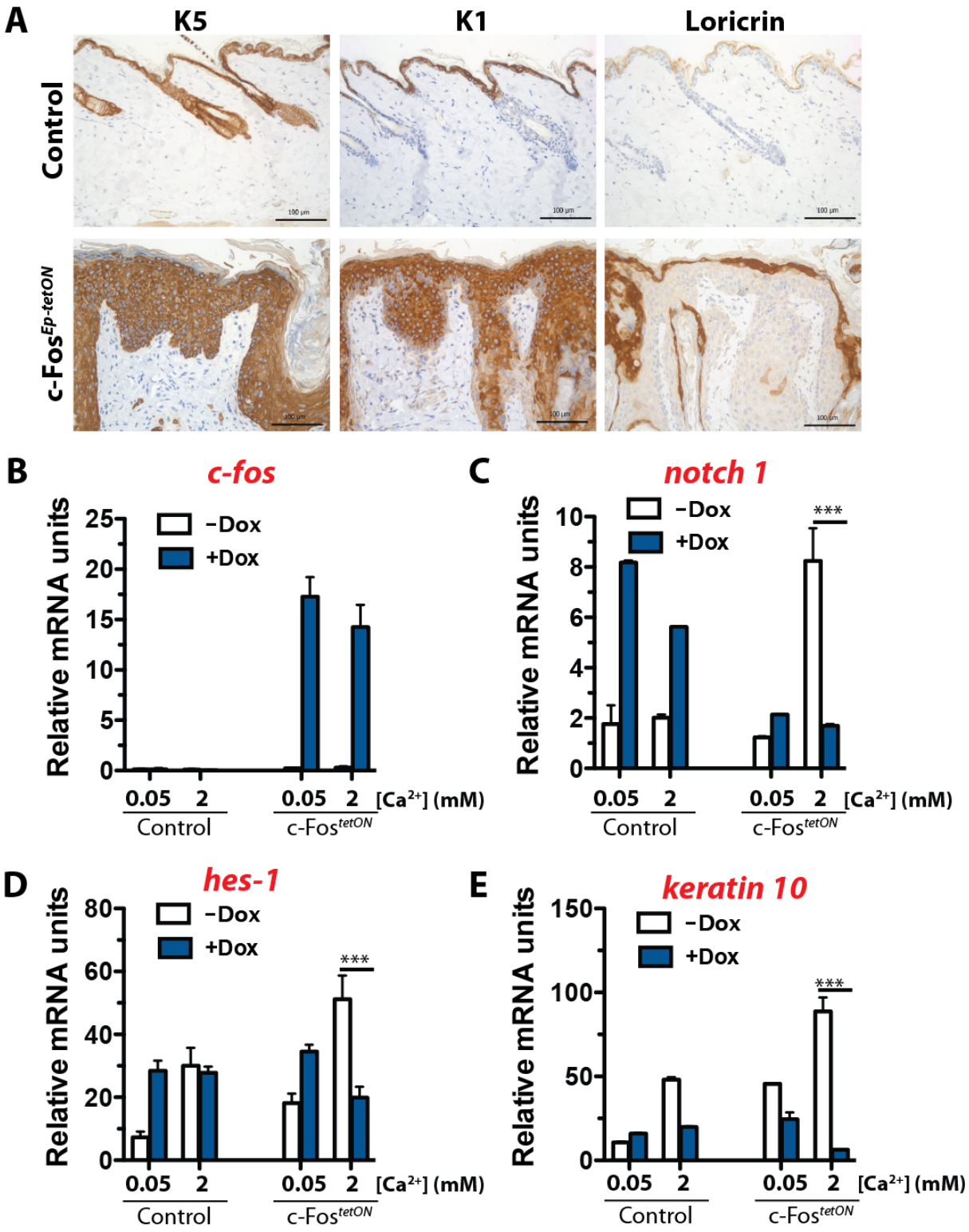
1.1.3. Impaired differentiation upon *c-fos* expression *in vitro*

Previous studies in our lab have shown that *c-fos* expression in keratinocytes can impair the differentiation program by repressing TACE expression through p53 (Guinea-Viniegra et al., 2012). TACE, also known as TNF-α Converting Enzyme (or ADAM17), activates the Notch signaling pathway in keratinocytes by cleaving the extracellular domain of Notch (Guinea-Viniegra et al., 2012). Upon cleavage, the intracellular domain of Notch (NICD) translocates to the nucleus and activates several genes involved in keratinocyte differentiation.

Epidermal differentiation marker analysis *in vivo* in the c-Fos$^{Ep\text{-}tetON}$ mouse model upon 4 weeks of Dox treatment revealed a dramatic expansion of basal and suprabasal epidermal layers in mutant mice. Multiple cell layers were positive for K5 (Fig.5A) and K14 (not shown), which mark the basal cell compartment. Similarly, the spinous (K1 and K10) and the granular (filaggrin and loricrin) layers were expanded (Fig. 5A and data not shown), indicating that *c-fos* expression affects early and late keratinocyte differentiation.

To assess whether keratinocyte differentiation is impaired upon *c-fos* expression *in vitro*, we challenged primary control and c-Fos^{tetON} keratinocytes with 2mM calcium to induce differentiation. 24h after calcium treatment and Dox treatment, the levels of *notch1* and the levels of the transcription factor *hes1*, which is downstream of Noctch-1, did not increase as compared to controls (Fig. 5C and 5D). Furthermore, the levels of early differentiation markers *keratin10* and *keratin1* (Fig. 5E and data not shown) did not increase either. Moreover, *loricrin*, a late differentiation marker, showed not significant, but reduced levels upon c-Fos expression and calcium treatment (data not shown). These data support the fact that c-Fos interferes with keratinocyte differentiation programs as previously described (Guinea-Viniegra et al., 2012).

Figure 5.



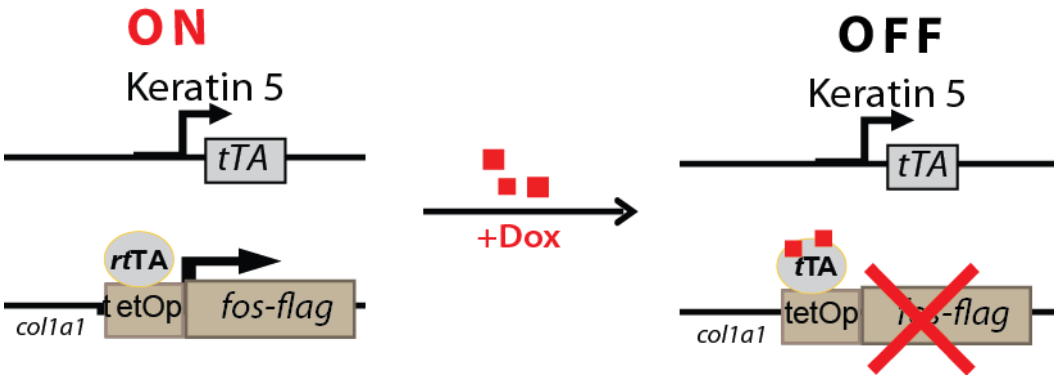
(A) Histological analyses of Keratin 5 (K5), Keratin 1 (K1) and Loricrin in back skin of c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks on Dox. (n=4). (B)(C)(D)(E) RT-qPCR analyses of total c-*fos, notch1, hes-1* and *keratin1* respectively mRNA levels in c-Fos^{tetON} keratinocytes treated ±Dox, +0.05/2mM Ca2+ for 24h. *p<0.05, ***p<0.0005.

1.2. Inducible keratinocyte-specific c-fos expression: c-Fos$^{Ep\text{-}tetOFF}$ mouse model

To validate the findings observed in c-Fos$^{Ep\text{-}tetON}$ mouse model upon inducible expression of *c-fos*, I generated another inducible mouse line, the c-Fos$^{Ep\text{-}tetOFF}$ mouse line. In this mouse model, we can induce expression of *c-fos* by removing the Dox treatment (Fig. 6). In our experimental set-up, mice are kept in Dox until their 6th or 8th week of age, when they are adults, and then Dox is removed to induce *c-fos* expression (Fig. 7A).

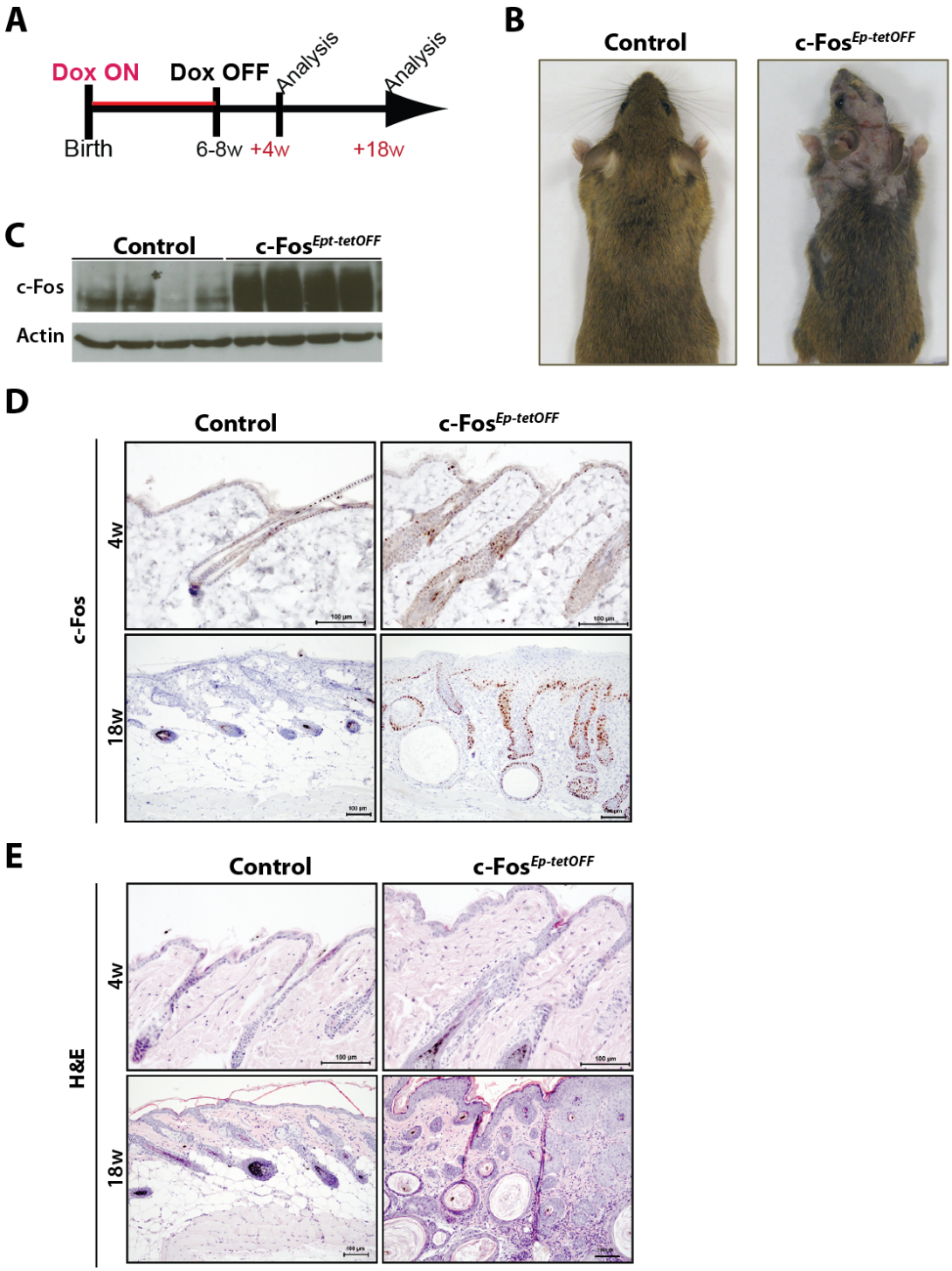
Upon *c-fos* expression in K5-expressing tissues using the tet-OFF system, the same features were recapitulated as in the c-Fos$^{Ep\text{-}tetON}$ mouse model, such as hair loss and development of scaly plaques (Fig. 7B). Increased c-Fos protein levels were detected in back skin of mice by immunoblot (Fig. 7C) and immunohistochemistry (Fig. 7D). Histologically, 18 weeks after Dox removal, the skin of mutant mice developed epidermal hyperplasia, hair follicle cysts and increased proliferation (Figure 7E and data not shown). These data confirmed the findings obtained using the c-Fos$^{Ep\text{-}tetON}$ mouse line. However, the development of the phenotype was delayed and the mice died later compared to the c-Fos$^{Ep\text{-}tetON}$ mouse line, probably due to the differences in the levels of inducible *c-fos* expression when using the two different lines (K5-tTA and K5-rtTA mouse lines).

Figure 6.



c-Fos$^{Ep\text{-}tetOFF}$ mouse model. Dox is supplied in food pellets. Upon Dox treatment, the rtTA binds to the tetOP activating the expression of exogenous *c-fos* in K5-expressing tissues.

Figure 7.



(A) Experimental set-up in c-Fos$^{Ep\text{-}tetOFF}$ mice. (B) Pictures of c-Fos$^{Ep\text{-}tetOFF}$ mice after 18 weeks off Dox. (C) Immunoblot depicting c-Fos total levels in back skin extracts of c-Fos$^{Ep\text{-}tetON}$ mice after 18 weeks off Dox. (D) Histological analyses of c-Fos in back skin of c-Fos$^{Ep\text{-}tetOFF}$ mice after 4 and 18 weeks off Dox. (n=3/3, 3/4). (E) H&E stainings of back skin of c-Fos$^{Ep\text{-}tetOFF}$ mice after 4 and 18 weeks off Dox. (n=3/3, 3/4)

1.2.1. The epidermal barrier is not affected upon *c-fos* expression

The epidermis is an effective barrier that prevents dehydration from loss of body water, poisoning from the absorption of noxious substances and systemic infection from invading surface microorganisms (Elias, 2007). When the skin barrier is disrupted, the body is more susceptible to infections and develops skin inflammatory and hyperproliferative diseases such, as atopic dermatitis and psoriasis (Hogan et al., 2012; Sonnenberg et al., 2011).

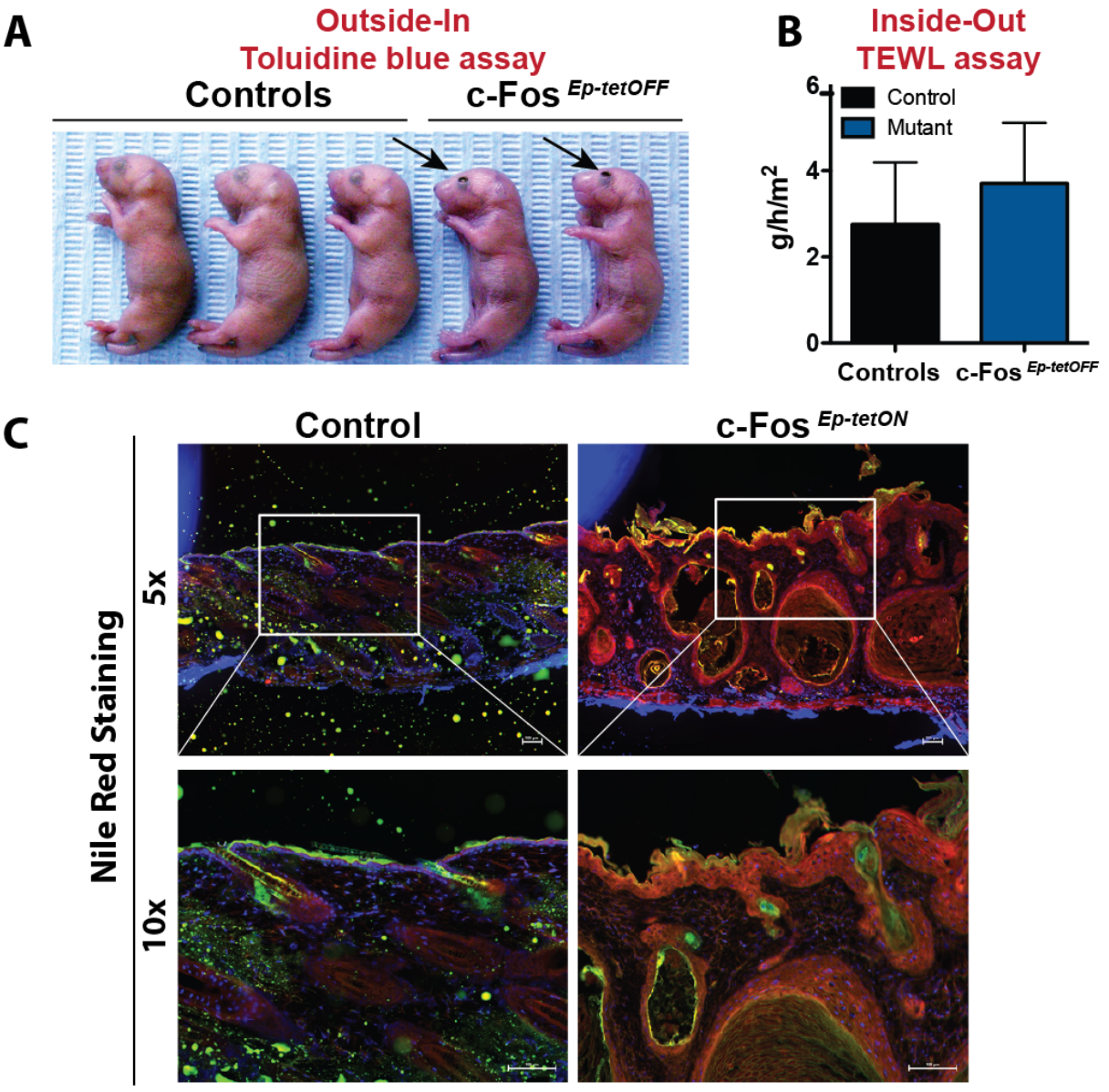
We therefore tested whether increased epidermal c-Fos levels could interfere with the proper development of the epidermal barrier by performing different assays. First, I tested the integrity of the outside-in barrier. We used P1 c-Fos$^{Ep\text{-}tetOFF}$ pups, which were bred without Dox (expression of *c-fos* is induced), and performed a toluidine blue penetration assay. We used P1 c-Fos$^{Ep\text{-}tetOFF}$ pups, since at this stage the pups have no hair and the procedure is easily performed. However, we did not observe any penetration of the dye in the skin of the pups (Fig. 8A), as it occurs when the integrity of the barrier is disrupted and the dye penetrates the skin. Interestingly, these mice displayed open eyes at birth (Fig. 8A, arrows). The same phenotype is observed when the expression of other AP-1 members in the epidermis, such as c-Jun and JunB, is perturbed (Zenz et al., 2003; Guinea-Viniegra et al., 2009).

The second most commonly used method to measure the integrity of the epidermal barrier is to test the quantity of water that evaporates from inside of the body through the epidermal layer to the outside of the body and this test is called Transepidermal Water Loss (TEWL) (Verdier-Sevrain and Bonte, 2007). When the barrier is disrupted, increased evaporation is measured. I performed this test using P1 c-Fos$^{Ep\text{-}tetOFF}$ pups, which were bred without Dox and we did not observe significant differences between controls and mutants (Fig. 8B).

An additional method to assess the integrity of the epidermal barrier is to assess the lipid matrix deposition in the epidermis. For this purpose, we used the c-Fos$^{Ep\text{-}tetON}$ mouse model. Frozen back skin sections from mice kept on Dox for 4 weeks were stained with Nile Red dye. Nile Red is a dye that fluoresces yellow-gold in the presence of neutral lipids (found in the *stratum corneum*) and red in the presence of polar lipids (found in the granular and spinous layer). No significant changes in the lipid content

were observed between controls and c-Fos$^{Ep\text{-}tetON}$ mice (Fig. 8C). Altogether these data indicate that increased levels of c-Fos either during development or during adulthood do not interfere with the development or impair the integrity of the epidermal barrier function.

Figure 8.



(A) Picture depicting 5 P1 pups (3 controls and 2 c-Fos$^{Ep\text{-}tetOFF}$) after toluidine blue penetration assay. Arrows indicate open eyes at birth and tail clip as a positive control. (n=10/5).(B) Transepidermal Water Loss (TEWL) measurements in P1 control and c-Fos$^{Ep\text{-}tetOFF}$ pups. (n=10/5). (C) Nile Red staining to measure lipid deposition in the epidermis. Red color: polar lipids. Yellow color: non polar lipids. (n=4).

2. *Mmp10* AND *s100a7a15* ARE TWO NOVEL TRANSCRIPTIONAL TARGET GENES OF c-FOS

2.1. Genome-wide expression analyses revealed novel target genes of c-Fos in keratinocytes

To molecularly understand the phenotype developed upon inducible *c-fos* expression in the epidermis, primary *in vitro* cultured c-Fos^{tetON} keratinocytes were employed for identification of novel transcriptional targets in keratinocytes.

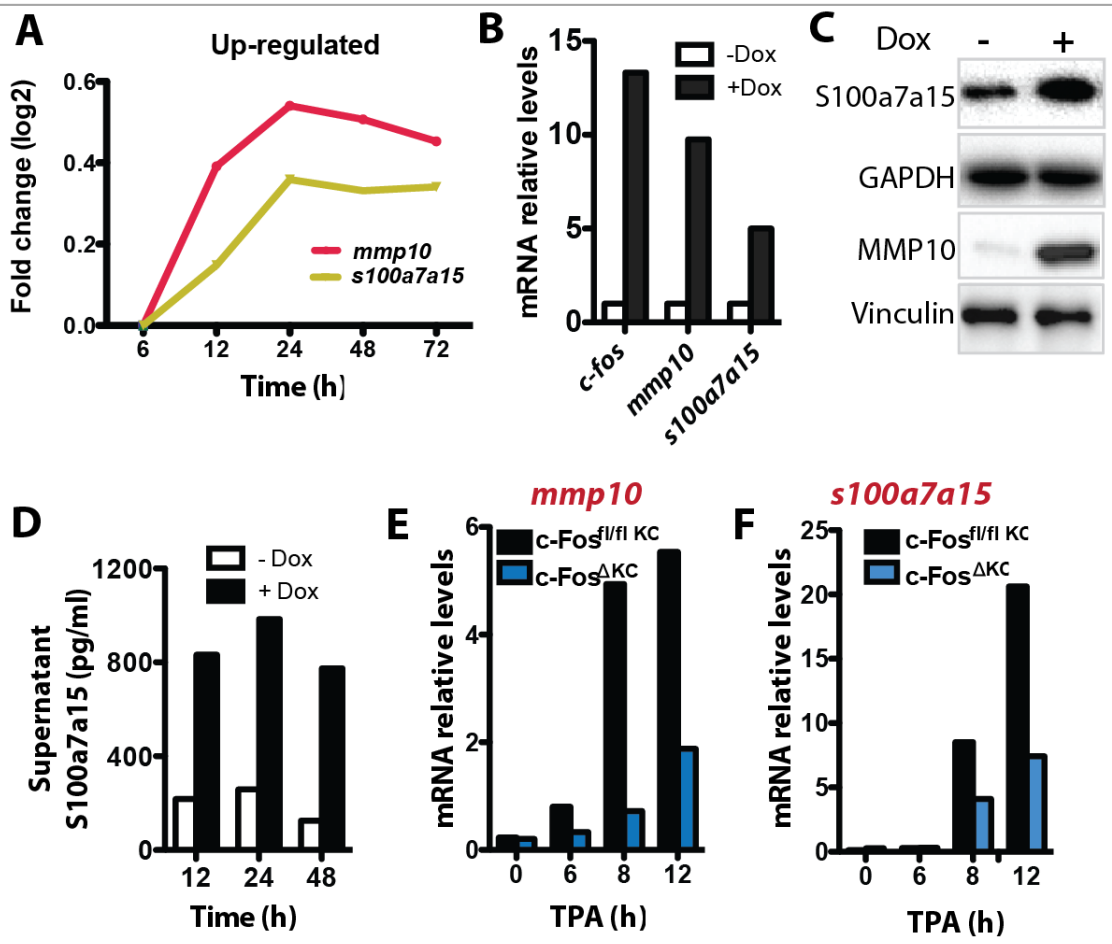
Genome-wide expression analyses using mutant primary keratinocytes were performed in collaboration with Peter Angel (DKFZ, Germany) to compare *c-fos* expressing *versus* non-expressing keratinocytes (±Dox) for 6, 12, 24, 48 and 72h. Interestingly, no major changes in the expression of genes involved in proliferation or differentiation were observed. However, among a number of c-Fos-induced genes, we found two consistently up-regulated genes, *mmp10*, a matrix metalloprotease involved in extracellular matrix remodeling and *s100a7a15*, a chemo-attractant molecule for CD4^{+}T lymphocytes as well as granulocytes (Fig. 9A).

2.2.Validation of c-Fos target genes *in vitro*

The expression of these two novel potential target genes, identified by means of genome-wide expression analyses, was validated at the RNA and protein level *in vitro.* A significant increase in the levels of mRNA (Fig. 9B) and protein by immunoblot and ELISA, (Fig. 9C, D) of *mmp10* and of *s100a7a15* was observed upon of *c-fos* induction *in vitro*.

To confirm that these genes are putative c-Fos target genes, I tested expression of both genes in *c-fos*-deficient keratinocytes. First, I stimulated keratinocytes with TPA (12-O-Tetradecanoylphorbol 13-acetate), an AP-1 agonist, to induce expression of *c-fos,* and a significant increase in the expression of both *mmp10* and *s100a7a15* was detected. However, this increase was reduced in c-Fos-deficient keratinocytes (Fig. 9E and 9F). These data suggest that *mmp10* and *s100a7a15* are likely to be transcriptionally regulated by c-Fos.

Figure 9.



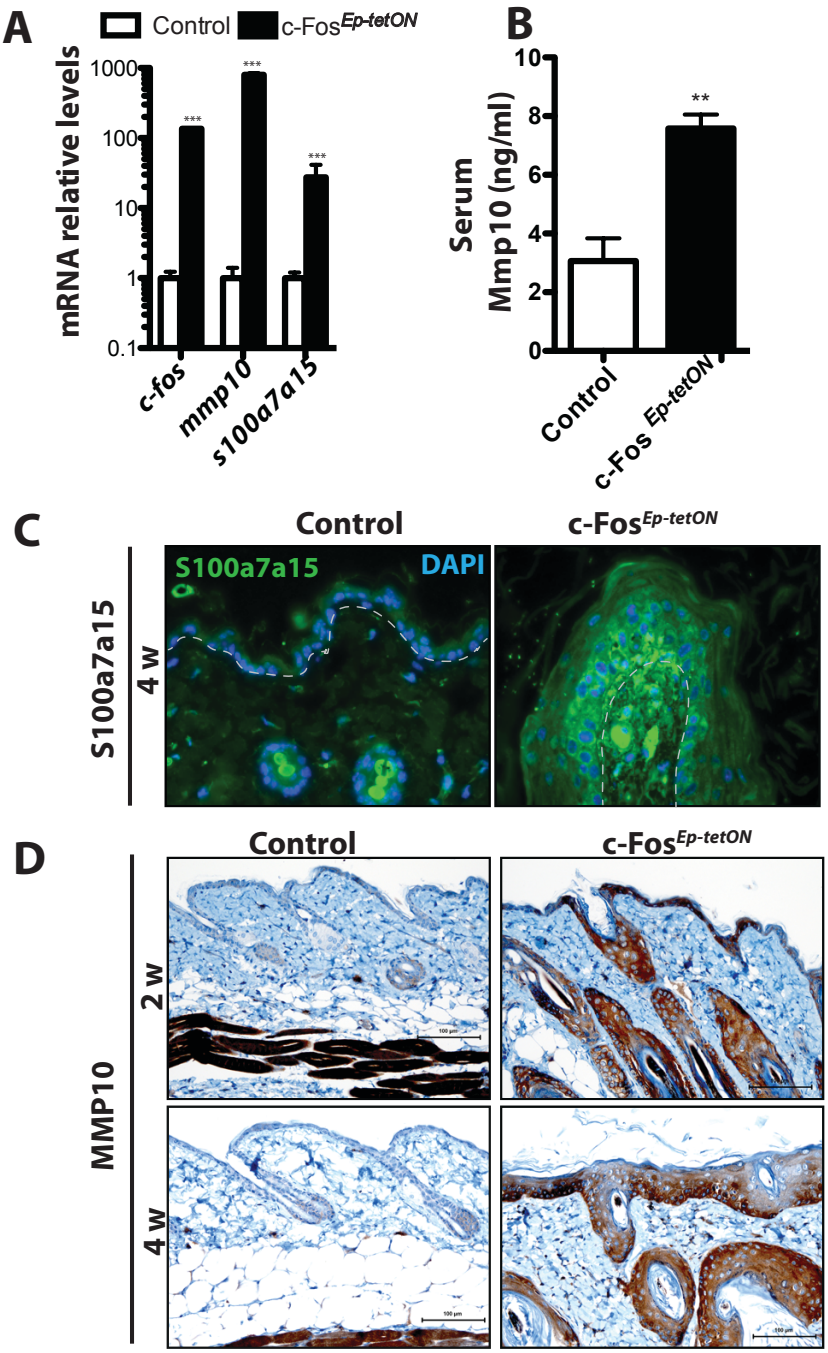
(A) Genome-wide expression analyses showing 2 consistently up-regulated genes in c-Fos^{tetON} keratinocytes comparing cells treated ±Dox (±*c-fos* expression) after 6 to 72 hours of culture. (B) RT-qPCR analyses of c-fos, *mmp10* and *s100a7a15* expression in c-Fos^{tetON} keratinocytes ±Dox after 6 to 72hours of Dox treatment. (C) Immunoblot of S00a7a15, MMP10 and the house keeping genes, GAPDH and Vinculin after 24h ±Dox treatment in c-Fos^{tetON} keratinocytes. (D) S100a7a15 protein levels measured in supernatants of c-Fos^{tetON} keratinocytes treated for 12, 24 and 48 hours ± Dox. (E,F) *In vitro* mRNA analyses of *mmp10* (E) and *s100a7a15* (F) levels in c-Fos$^{fl/fl}$ keratinocytes treated either with Ad-Cre (blue bar) o Ad-GFP (black bar).

2.3. Validation of c-Fos target genes *in vivo*

We next assessed the expression of *mmp10* and *s100a7a15* in the back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice. We detected increased *mmp10* and *s100a7a15* mRNA levels in the epidermis of c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks of *c-fos* induction, as analyzed by RT-qPCR (Fig. 10A). Moreover, increased levels of MMP10 protein were detected in the sera of c-Fos$^{Ep\text{-}tetON}$ mice measured by ELISA at 2 weeks of Dox treatment (Fig. 10B). Histological (IHC and immunofluorescence) analyses also confirmed elevated levels of

these two proteins in the back skin of c-Fos$^{Ep\text{-}tetON}$ mice after 2 and 4 weeks of inducible *c-fos* expression (Fig. 10C, 10D). These data suggest that c-Fos could be a transcriptional regulator of *mmp10* and *s100a7a15.*

Figure 10.

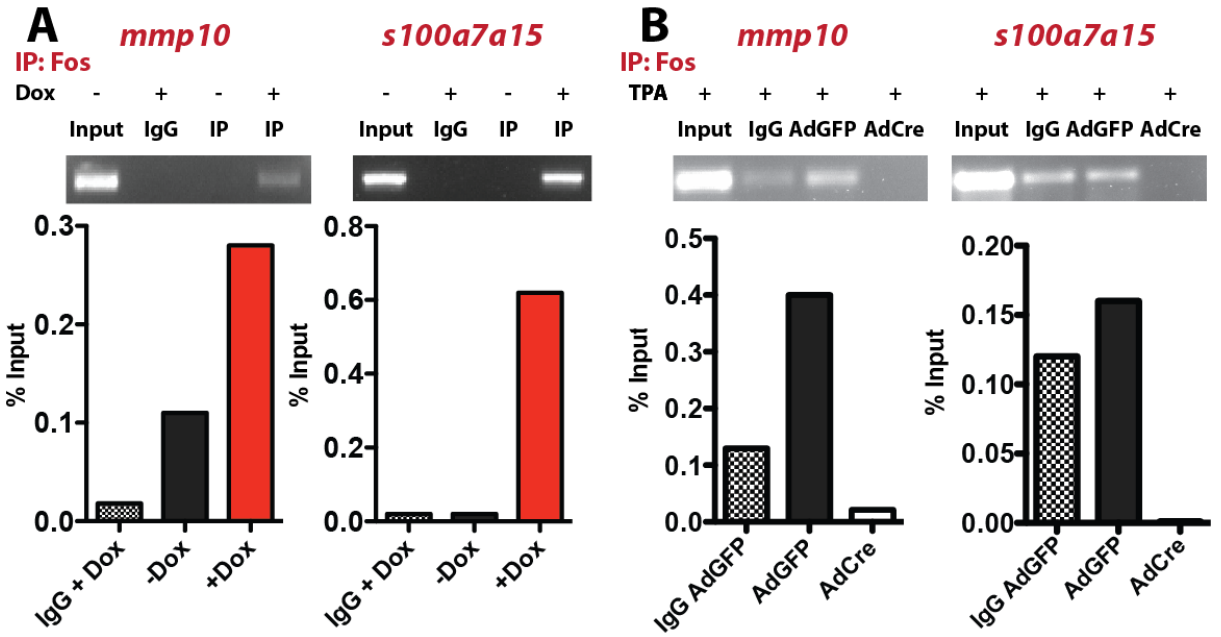


(A) RT-qPCR analyses of *c-fos, mmp10* and *s100a7a15* expression of back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks of Dox treatment. (B) MMP10 levels in the serum of c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks of Dox treatment. (C) Immunofluorescence analyses of S100a7a15 in back skin of c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks of Dox treatment. (D) Immunohistochemical analyses of MMP10 in the back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice after 2 and 4 weeks of Dox treatment.

2.4. *Mmp10* and *s100a7a15* promoter analyses

To ascertain whether c-Fos transcriptionally controls *mmp10* and *s100a7a15* expression, I assessed c-Fos recruitment to their promoters by chromatin immunoprecipitation (ChIP) assays. The promoter regions of *mmp10* and *s100a7a15* contain 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE) to which AP-1 family of transcripction factors can bind. c-Fos was found bound to both *mmp10* as well as *s100a7a15* promoters in primary c-Fos$^{Ep\text{-}tetON}$ keratinocytes, when *c-fos* expression was induced by Dox treatment for 24h (Fig. 11A). Furthermore, ChIP analyses using *c-fos*$^{fl/fl}$ keratinocytes transduced with Adenoviral-Cre (termed as c-Fos$^{\Delta Kc}$) or Adenoviral-GFP (termed as c-Fos$^{fl/fl}$) and induced with TPA (an AP-1 agonist) for 3 hours were performed. We observed that upon *in vitro c-fos* deletion, no binding to *mmp10* and *s100a7a15* promoters was detected (Fig. 11B). These results indicate that *mmp10* and *s100a7a15* are direct transcriptional targets of c-Fos.

Figure 11.



(A) Chromatin immunoprecipitation (ChIP) of c-Fos at the *mmp10* (left) and *s100a7a15* (right) promoters. Endpoint RT-qPCR-fragments are shown together with the representation of % of binding of c-Fos to *mmp10* and *s100a7a15* promoters. Chromatin was immunoprecipitated using c-Fos antibody from c-Fos^{tetON} keratinocytes treated ±Dox for 24h. (B) ChIP of c-Fos at the *mmp10* and *s100a7a15* promoters. Endpoint qPCR-fragments are shown together with the representation of % of binding of c-Fos to *mmp10* (left) and *s100a7a15* (right) promoters. Chromatin was immunoprecipitated using c-Fos antibody in c-Fos$^{fl/fl}$ and c-Fos$^{\Delta Ep}$ keratinocytes treated for 3h with TPA.

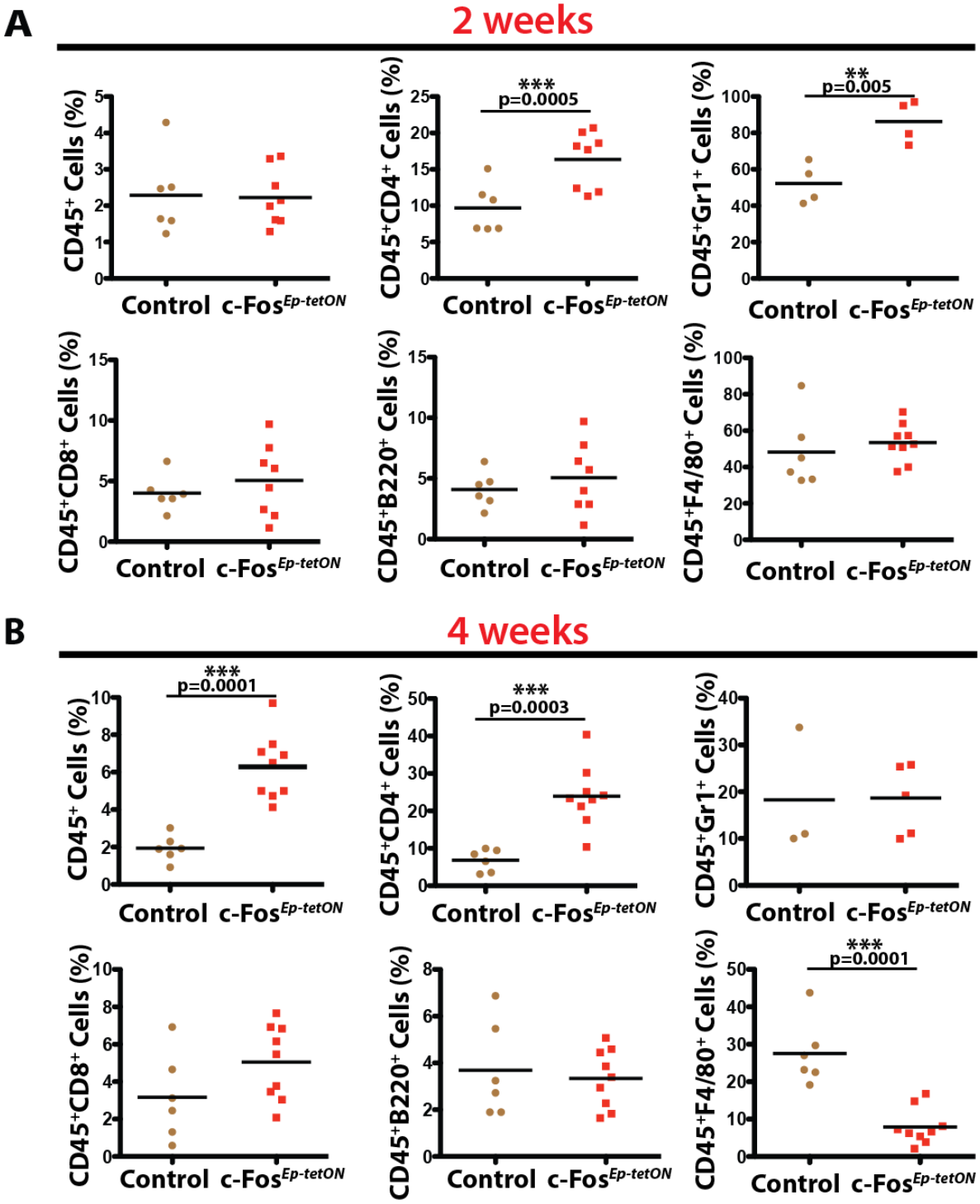
3. *c-fos* EXPRESSION INDUCES SKIN INFLAMMATION CHARACTERIZED BY CHRONIC CD4 T CELL RECRUITMENT

S100a7a15 has been shown to be involved in the recruitment of CD4 T cells and granulocytes (Wolf et al., 2008). MMP10 can also facilitate the recruitment of leukocytes from the bloodstream by degrading components of the extracellular matrix and by modulating cytokine and chemokine activity (Elkington et al., 2005). Moreover, inflammatory cells invade tumors, and these cells are a source of growth factors and cytokines that facilitate tumorigenesis (Coussens et al., 1999; Hanahan and Coussens, 2012). We therefore examined the presence of inflammatory cells in the skin of c-Fos$^{Ep\text{-}tetON}$ and control mice over time that would explain the preneoplastic phenotype since proliferation occurred in a non-cell autonomous manner.

Flow cytometry analyses of back skin at 2 weeks after inducible expression of c-Fos revealed no significant changes in total leukocyte infiltration, as analyzed with the pan leukocyte marker CD45. However, CD4 T cell, as well as Gr1 granulocyte number were already significantly increased at this time point (Fig. 12A). No changes were observed in the infiltration of macrophages, B cells and CD8 T cells (Fig. 12A). Moreover, at 4 weeks, when the epidermis is already hyperplastic, we found substantial leukocyte infiltration (assessed by CD45 staining) (Fig. 12B). Interestingly, there was a chronic and pronounced accumulation of CD4 T cells in the skin of mutant mice at this time point but no changes in Gr1$^+$ cell number was observed (Fig. 12B). These cells expressed high levels of CD44 protein, a marker of CD4 T cell activation (Fig. 13A). These data indicate that chronic recruitment of CD4 T cells could be detrimental for the epidermal homeostasis and could likely be the cause of the phenotype developed upon *c-fos* expression in the epidermis.

Analyses of the activation status of CD4 T cells in the draining lymph nodes revealed increased CD4$^+$CD44high cells in c-Fos$^{Ep\text{-}tetON}$ mice compared to control mice (Fig. 13B). Therefore, the accumulation of CD4 T cells in the skin of mutant mice is likely the result of the recruitment of activated CD4 T cells from draining lymph nodes, instead of an expansion of resident CD4 T cells in the homeostatic skin.

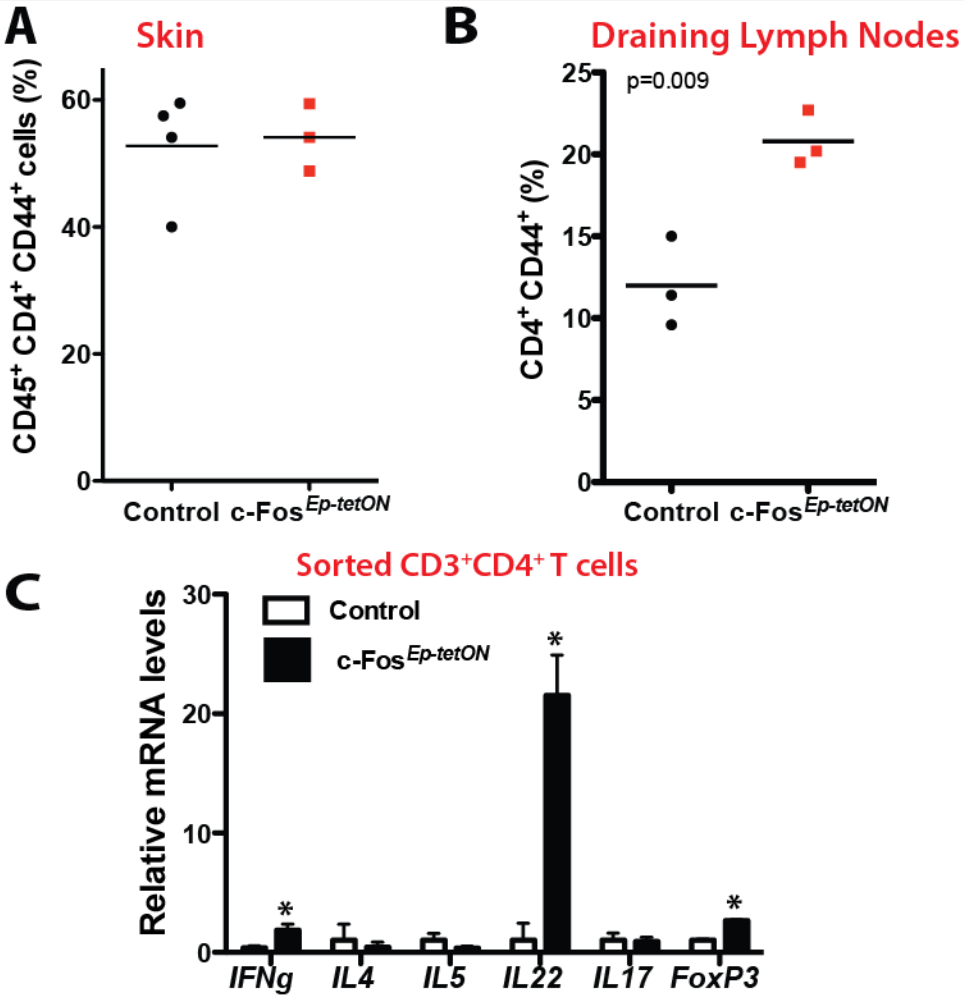
Figure 12.



(A) Flow cytometry analyses of the skin immune-cell infiltrate in 6-8 week-old control and c-Fos$^{Ep\text{-}tetON}$ mice (n=6/9) after 2 weeks of ectopic *c-fos* expression. (B) Flow cytometry analyses of the skin immune-cell infiltrate in 6-8 week-old control (n=6) and c-Fos$^{Ep\text{-}tetON}$ (n=9) mice at 4 weeks of ectopic *c-fos* expression.*p<0.05, **p<0.005, ***p<0.0005.

To analyze which type of effector CD4 T cells penetrate the skin of c-Fos$^{Ep\text{-}tetON}$ mice, CD4$^+$CD3$^+$ T cells were FACS sorted from the skin after 4 weeks of inducible *c-fos* expression. I examined the expression of INF-γ, as a functional marker for Th1 cells, IL-4 and IL-5 as markers of the Th2 lineage, IL-17, as a marker of the Th17 cell lineage, FoxP3, as a marker for Treg cells, and IL-22, as a marker of the Th22 cell lineage (Fig. 13C). Overall there were no significant changes in the expression levels of most cytokines. Interestingly, there was a marginal increase in the levels of Fox P3 and a striking elevation of IL-22 expression in CD4 T cells from mutant mice compared to controls.

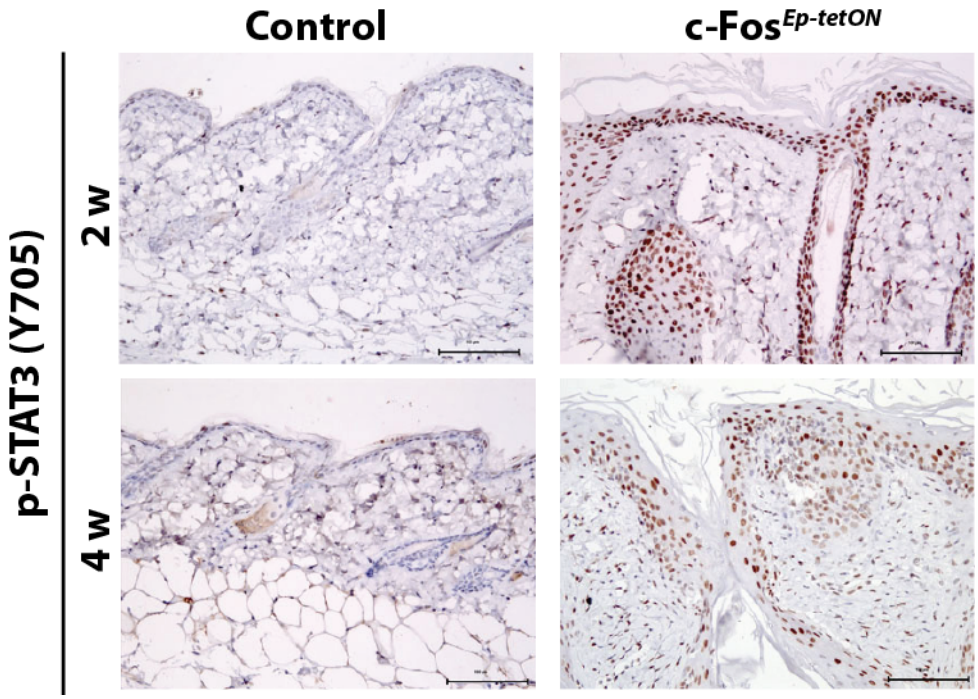
Figure 13.



(A) Flow cytometry analyses of the immune-cell infiltrate in the skin of 6-8 week-old control and c-Fos$^{Ep\text{-}tetON}$ (n=3) mice at 2 weeks of ectopic c-fos expression. (B) Flow cytometry analyses of the immune-cell infiltrate in the draining lymph nodes of 6-8 week-old control and c-Fos$^{Ep\text{-}tetON}$ (n=3) mice at 2 weeks of ectopic c-fos expression. (C) RT-qPCR Expression analyses of CD3$^+$CD4$^+$ sorted T lymphocytes from the back skin of 6-8 week-old control and c-Fos$^{Ep\text{-}tetON}$ mice after 4 weeks of inducible *c-fos* expression (n=3). *P<0.05

IL-22 has been implicated in the control of tumor growth and progression (Liu et al., 2012; Curd et al., 2012) as well as in skin inflammatory diseases, such as psoriasis (Pan et al., 2012). IL-22 signals through activation of STAT3 and gp130, a signal transducer associated with IL-22 receptor. Analysis of activation of STAT3 by IHC showed activated STAT3 (Y705P-STAT3) in the epidermis of c-Fos^{EptetON} mice compared to control mice 2 weeks after inducible *c-fos* expression (Fig. 14). Thus, IL-22 supplied by infiltrating CD4 T cells and recruited as a consequence of increased levels of MMP10 and S100a7a15, may contribute to keratinocyte survival and/or proliferation, eventually leading to epidermal hyperplasia as shown upon inducible *c-fos* expression. These data suggest a novel mechanism by which c-Fos-induced infiltration of CD4 T cells in the "primed" skin, leads to the release of specific cytokines, such as IL-22, which by activation of specific pathways in keratinocytes promote cell survival and proliferation.

Figure 14.



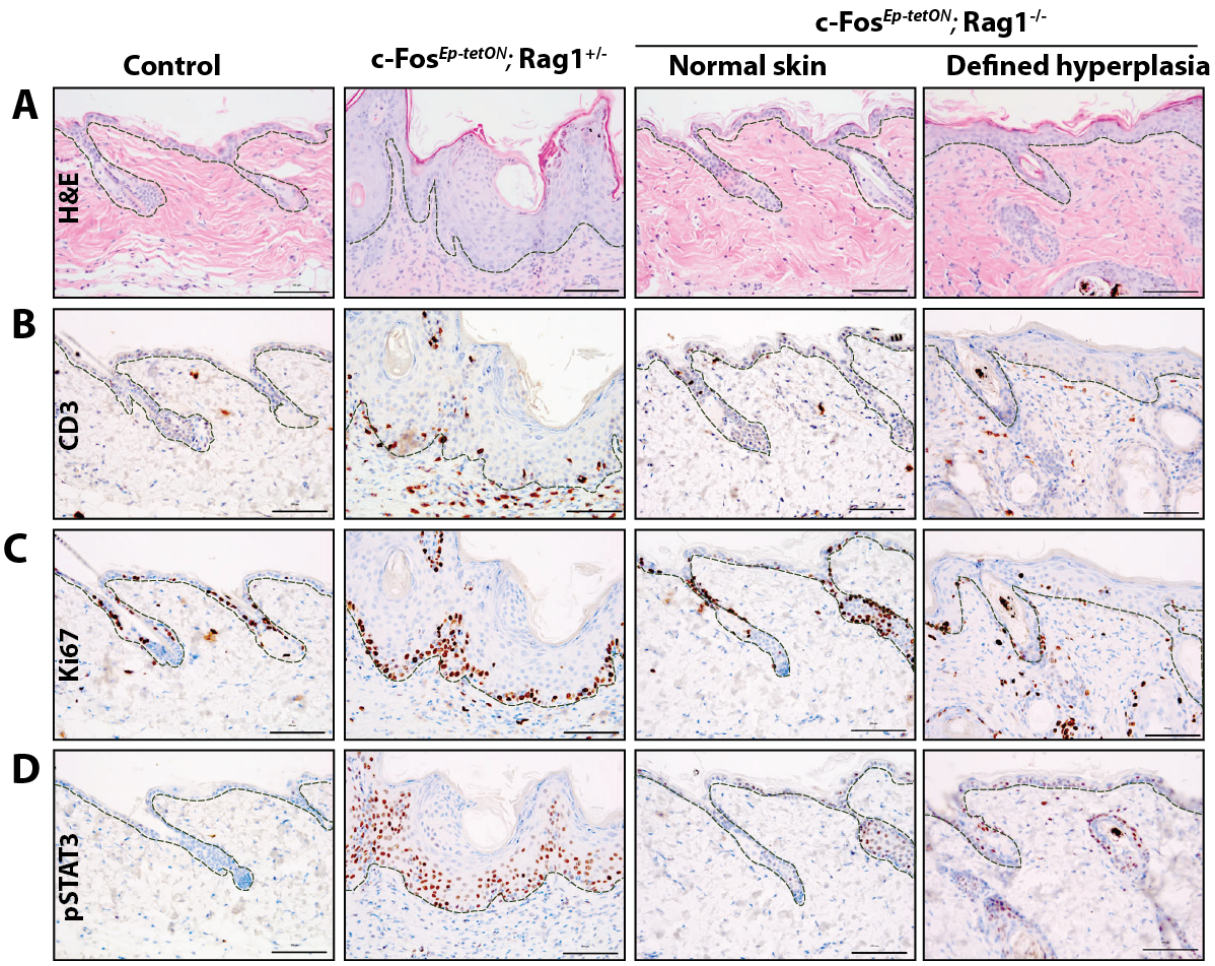
p-STAT3 (Y705) immunostaining in back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice after 2 and 4 of weeks ectopic *c-fos* expression (n=4/4/4/4).

4. INTERFERING WITH CD4 T CELL RECRUITMENT SIGNIFICANTLY IMPAIRS c-FOS-MEDIATED EPIDERMAL HYPERPLASIA

Previous studies by Coussens *et al* have shown that CD4 T cells heavily infiltrate preneoplastic lesions and SCC in the K14-HPV mouse model (Junankar et al., 2006). Moreover, genetic depletion of CD4 T cells in the same tumor-prone mouse model (K14-HPV) leads to a delayed neoplastic progression and a lower incidence of tumors (Daniel et al., 2003). Since chronic recruitment of CD4 T cells upon *c-fos* expression was observed, we genetically depleted this population of immune cells by crossing the c-Fos$^{Ep\text{-}tetON}$ mouse model to the RAG1$^{-/-}$ mice (Mombaerts et al., 1992). RAG1 and RAG2 (recombination-activating gene) are two proteins necessary for immunoglobulin and T-cell receptor gene recombination. RAG1 and RAG2 work closely together in receptor gene recombination and they are both necessary for V(D)J recombination (Schatz and Ji, 2011).

Interestingly, upon RAG1 deletion, in the absence of mature T and B cells, c-Fos$^{Ep\text{-}tetON}$ mice developed significantly smaller lesions (Fig. 15A), while some mice, displayed defined isolated regions of mild hyperplasia. Moreover, we assessed the levels of lymphocytes by performing CD3 immunostainings. As expected, no CD3^{+} cells were observed in RAG1 deficient mice (Fig. 15B). Interestingly, in c-Fos$^{Ep\text{-}tetON}$; Rag$^{-/-}$ mice, keratinocyte proliferation, assessed by Ki67 immunostaining, and p-STAT3, a marker for keratinocyte survival, were significantly reduced (Fig. 15C,D). These data confirm our hypothesis that CD4^{+}T cells are important mediators in the development of preneoplastic lesions.

Figure 15.

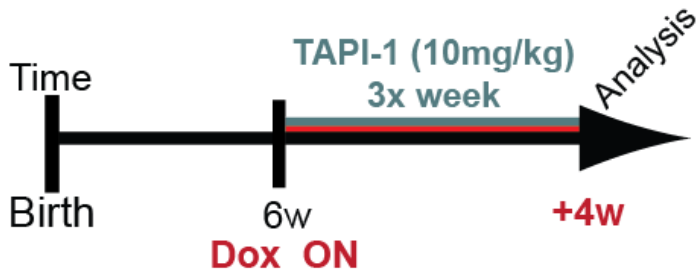


(A,B,C,D) Immunohistochemical analyses depicting H&E, CD3, Ki67, and pSTAT3 immunostainings, respectively, of back skin of control (Col-Fos (+/+); K5rtTA (+/+); Rag (-/-)), c-Fos$^{Ep\text{-}tetON}$ (Col-Fos (+/KI); K5rtTA (+/T); Rag (+/+)) an7 c-Fos$^{Ep\text{-}tetON}$ Rag-/- mice (Col-Fos (+/KI); K5rtTA (+/T); Rag (-/-) mice on Dox for 4 weeks (n=7/4/10, males).

5. BROAD MMP INHIBITION PREVENTS THE DEVELOPMENT OF PRENEOPLASTIC LESIONS UPON C-FOS EXPRESSION IN c-FOS$^{Ep\text{-}tetON}$ MICE

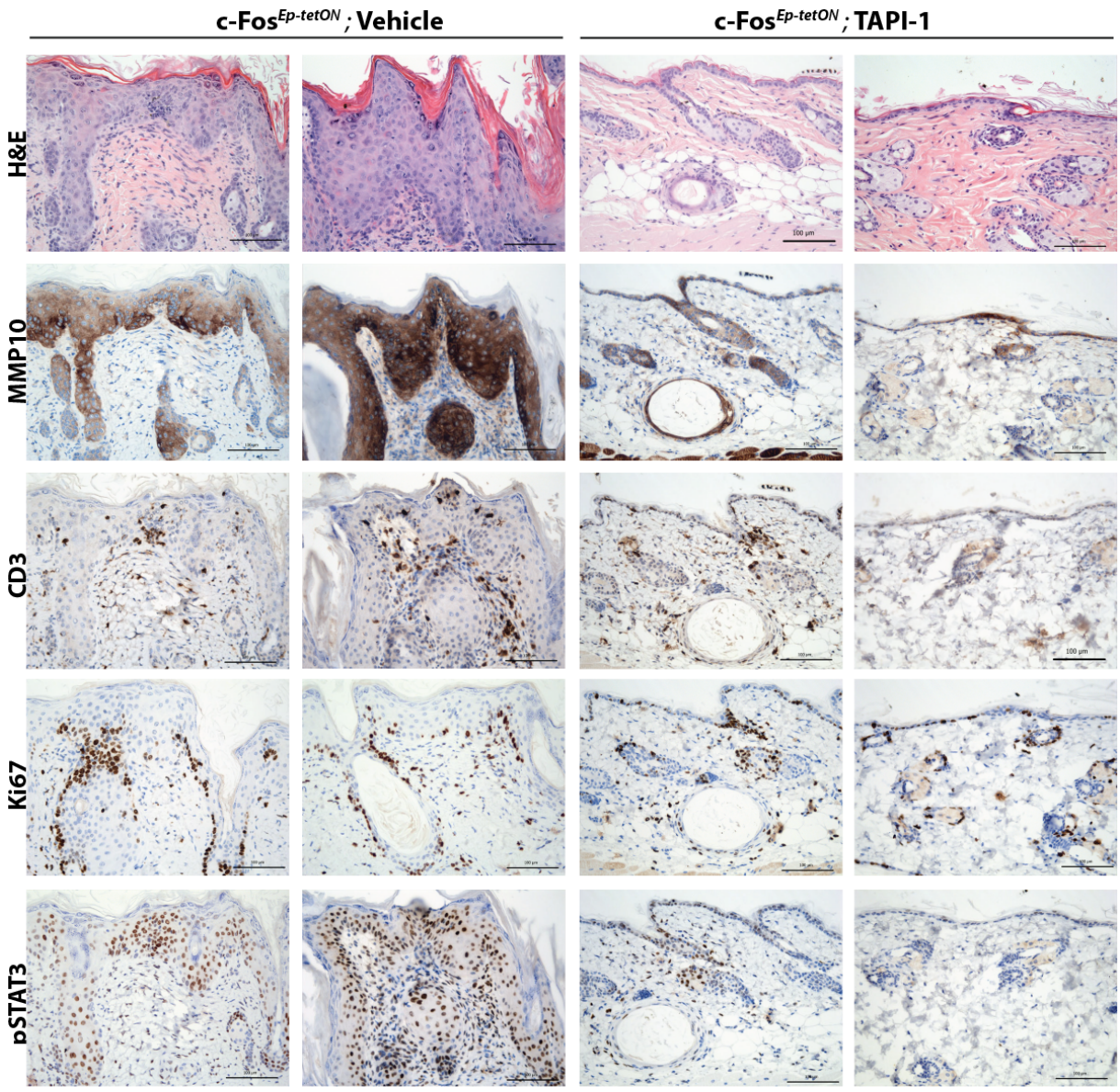
After confirming direct binding of c-Fos to the *mmp10* promoter and corroborating that *mmp10* is a direct transcriptional target of c-Fos, a broad MMP inhibitor (TAPI-1) was used *in vivo* to block MMP10 activity. The inhibitor was used as a preventive treatment and it was administered intraperitonally (IP) three times a week in parallel to the Dox treatment (Fig. 16). As assessed by H&E immunostainings, c-Fos$^{Ep\text{-}tetON}$ treated with the MMP inhibitor showed significant prevention in the development of the preneoplastic lesions (Fig.17). However, even though TAPI-1-treated c-Fos$^{Ep\text{-}tetON}$ mice did not show a dramatic hyperplasia, they presented other features commonly developed by c-Fos$^{Ep\text{-}tetON}$ mice, such as hair follicle cysts (Fig. 17). While c-Fos$^{Ep\text{-}tetON}$ mice still expressed high MMP10 protein levels upon TAPI-1 treatment, they showed no recruitment of T lymphocytes. Keratinocyte proliferation and survival, assessed by ki67 and p-STAT3 immunostaining respectively, were reduced compared to vehicle-treated mice. Nevertheless, MMP10 activity assays of epidermal protein lysates should be performed to assess the efficiency of this drug to reach the epidermis. These results indicate that inhibition of MMP signaling ameliorates the pathological features developed by the c-Fos$^{Ep\text{-}tetON}$ mice. However, not only a specific inhibitor should be developed but also further analyses should be performed to supply the drug subcutaneously and avoid systemic secondary effects.

Figure 16.



Experimental set-up. Control and c-Fos$^{Ep\text{-}tetON}$ mice were treated with Dox and with vehicle or 10mg/kg of TAPI-1 injected IP three times a week for 4 weeks.

Figure 17.



Immunohistochemical analyses depicting H&E, MMP10, CD3, Ki67, and pSTAT3 immunostainings respectively of back skin of c-Fos$^{Ep\text{-}tetON}$ mice (Col-Fos (+/KI); K5rtTA (+/T)) treated with vehicle or with TAPI-1 (10mg/kg, three times a week), (n=3/3).

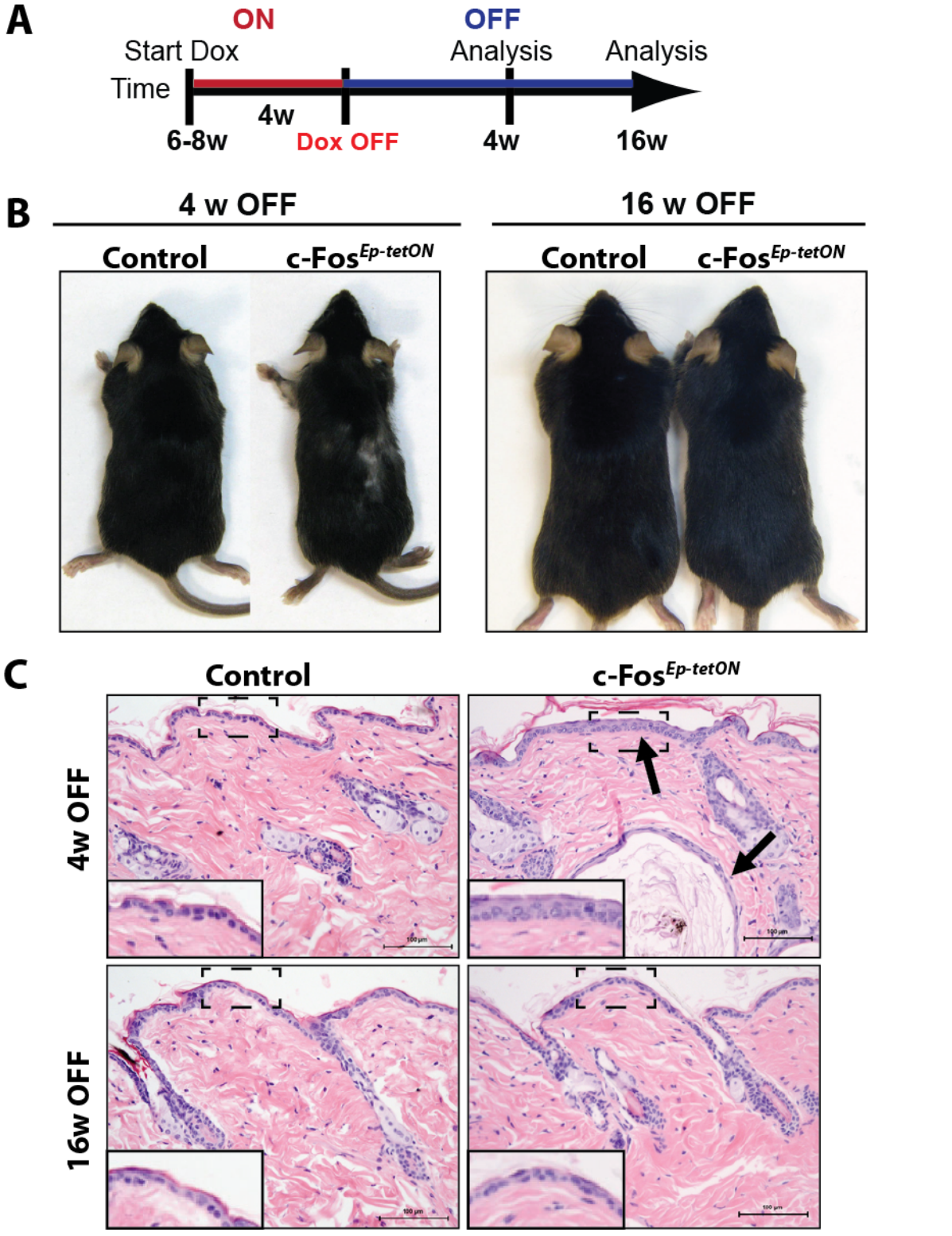
6. c-FOS-DEPENDENT SKIN PHENOTYPE IS LARGELY REVERSIBLE

To determine whether sustained expression of *c-fos* is required to maintain epidermal hyperplasia upon inducible *c-fos* expression, I made use of the switchable c-Fos$^{Ep\text{-}tetON}$ mouse model by removing Dox treatment. *c-fos* expression was induced for 4 weeks until the mice developed visible lesions at which point inducible *c-fos* expression was stopped for either 4 or 16 weeks (Fig. 18A). 4 weeks after switching off *c-fos* expression, c-Fos$^{Ep\text{-}tetON}$ mice almost fully recovered macroscopically, although 30% of the c-Fos$^{Ep\text{-}tetON}$ mice still contained areas where hair was absent (Fig. 18B). 16 weeks after stopping inducible *c-fos* expression, all mice had completely reverted the phenotype (Fig. 18C). Histologically, about 50% of the mice still showed areas of mild dysplasia as well as hair follicle cysts at 4 weeks, but not at 16 weeks (Fig. 18C, arrow).

IHC analyses were performed to analyze protein levels of c-Fos (not shown), Ki67 and MMP10 protein, which levels were still observed in the remaining dysplastic areas and in the cysts at 4 weeks after having stopped inducible *c-fos* expression, probably due to a slow turnover of the epidermis (Fig 19A,B). However, no expression of these markers was observed at 16 weeks (Fig. 19A,B). These results suggest an addictive effect of the epidermis to constant c-Fos signaling.

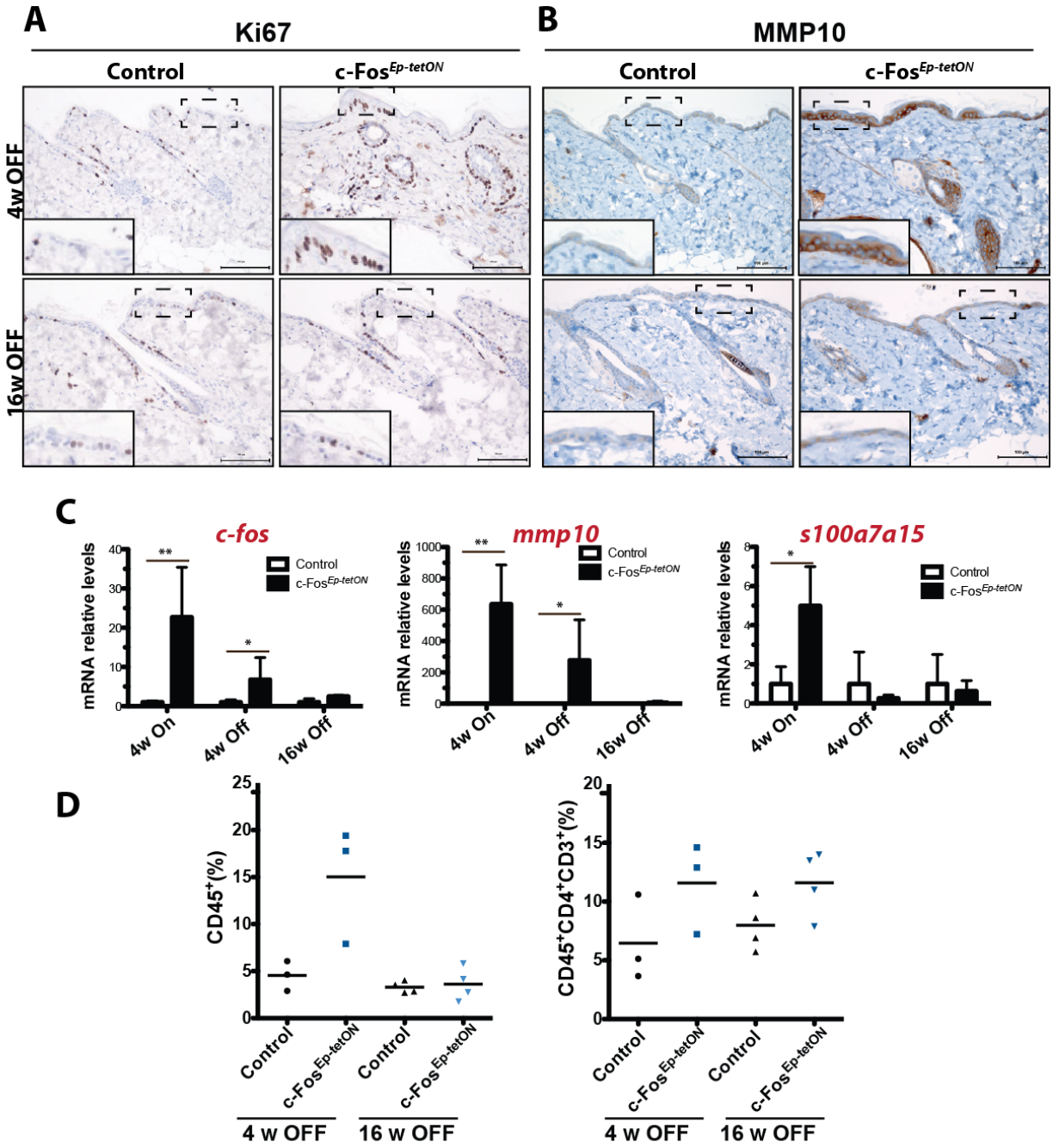
Expression analyses of total skin revealed that *c-fos* and *mmp10,* but not *s100a7a15* levels, remained high 4 weeks after stopping inducible *c-fos* expression (Fig. 19C) correlating with visible signs of epidermal architectural disruption. A slight increase, but not significant, in total leukocyte as well as CD4 T lymphocyte infiltration analyzed by flow cytometry was observed also 4 weeks after stopping inducible *c-fos* expression, but not after 16 weeks (Fig. 19D). These results indicate a clear dependence of *c-fos* expression, with CD4 T cell infiltration and the epidermal hyperproliferative status. Upon 16 weeks of inducible c-Fos removal, CD4 T cells are no longer recruited and as a consequence, keratinocyte proliferation is halted, as seen by Ki67 and phosphorylation of STAT3 (Fig. 19A and data not shown).

Figure 18.



(A) Experimental set-up. Control and c-Fos$^{Ep\text{-}tetON}$ mice are kept on Dox for 4 weeks and then the Dox treatment is removed for 4 or 16 weeks. (B) Picture of control and c-Fos$^{Ep\text{-}TetON}$ female mice after 4 weeks of Dox and 4 or 16 weeks off Dox administration. (C) H&E staining of normal skin from control and c-Fos$^{Ep\text{-}TetON}$ female on Dox for 4 weeks and Off Dox for 4 or 16 weeks of Dox administration. (n “4 weeks”=4/3, n “16 weeks”= 3/3).

Figure 19.



(A) Immunohistochemical analyses depicting Ki67 immunostainings of back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice treated on Dox for 4 weeks and kept without Dox for 4 or 16 weeks. (n=4/4). (B) Immunohistochemical analyses depicting MMP10 immunostainings of back skin of control and c-Fos$^{Ep\text{-}tetON}$ mice treated on Dox for 4 weeks and kept without Dox for 4 or 16 weeks. (n=4/4). (C) RT-qPCR expression *c-fos, mmp10* and *s100a7a15* of back skin of c-Fos$^{Ep\text{-}tetON}$ mice treated on Dox for 4 weeks and left without Dox for 4 or 16 weeks. (n=4/4). (D) Flow Cytometry analyses of the skin immune infiltrate of control c-Fos$^{Ep\text{-}tetON}$ mice treated on Dox for 4 weeks and left without Dox for 4 or 16 weeks. (n=4/4). *p<0.05, **p<0.005.

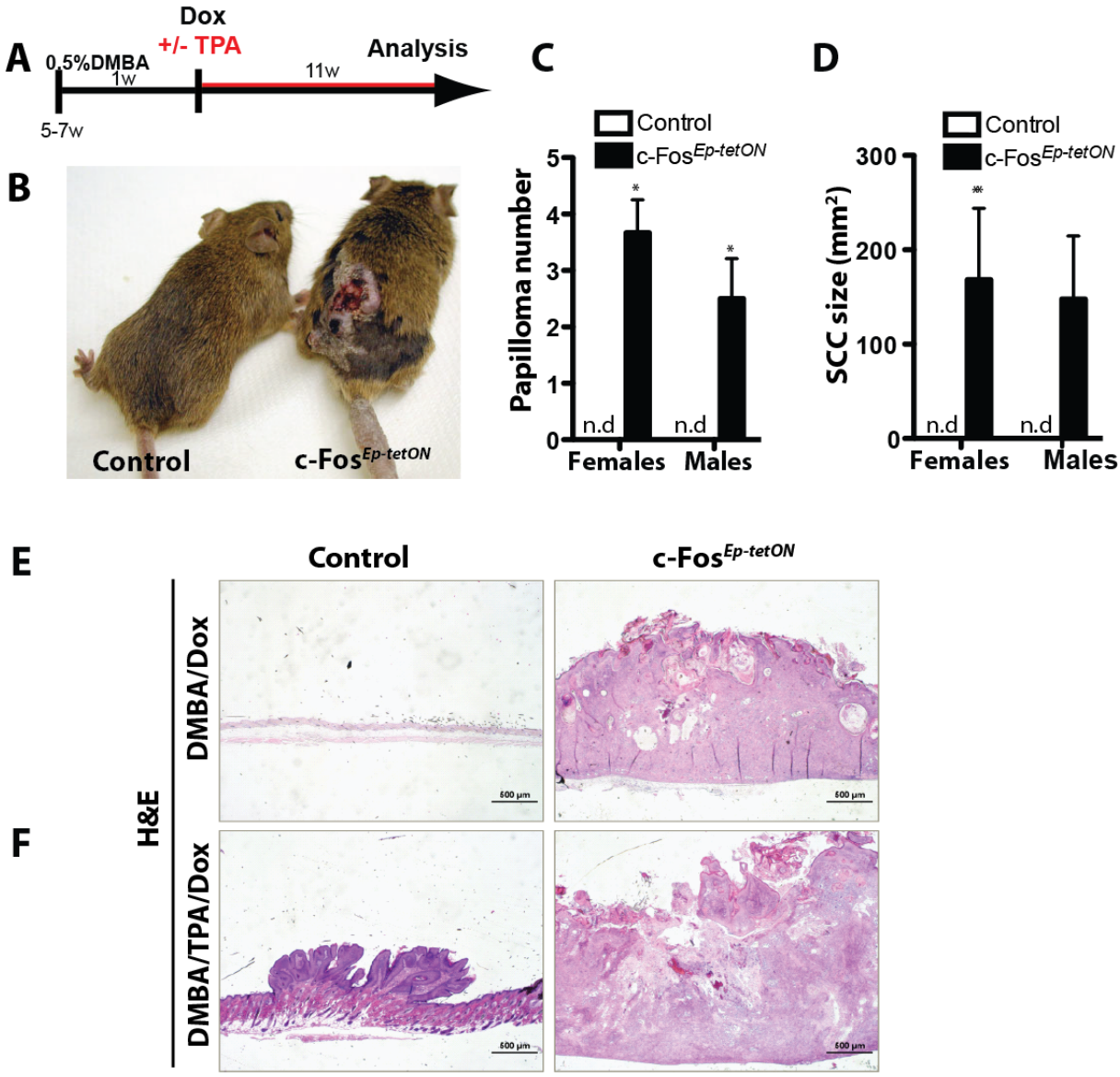
7. DMBA-INDUCED PAPILLOMA AND SCC DEVELOPMENT IS ACCELERATED BY c-FOS

7.1. c-Fos$^{Ep\text{-}tetON}$ mice develop invasive SCCs upon DMBA

To investigate the contribution of c-Fos to papilloma and tumor development, we applied an adapted version of the two-step skin carcinogenesis protocol (DMBA/TPA) to the c-Fos$^{Ep\text{-}tetON}$ mice (Kemp, 2005). H-Ras mutations were induced with DMBA in the back skin of adult mice and instead of TPA, *c-fos* expression was switched on 1 week later using a low dose of Dox (0.25g/l) (to slower the development of the skin phenotype and prevent lethality) (Fig. 20A). After 8 weeks of Dox treatment, mice developed papillomas (Fig. 20C) and at 11 weeks these lesions were identified as SCCs, whereas control mice did not develop any lesion (Fig. 20B, C, D). The tumor promoter TPA was included in a small number of animals that were also treated with DMBA and were given Dox treatment, as a positive control. In contrast, control mice after DMBA and 11 weeks of TPA developed benign papillomas (Fig. 20F).

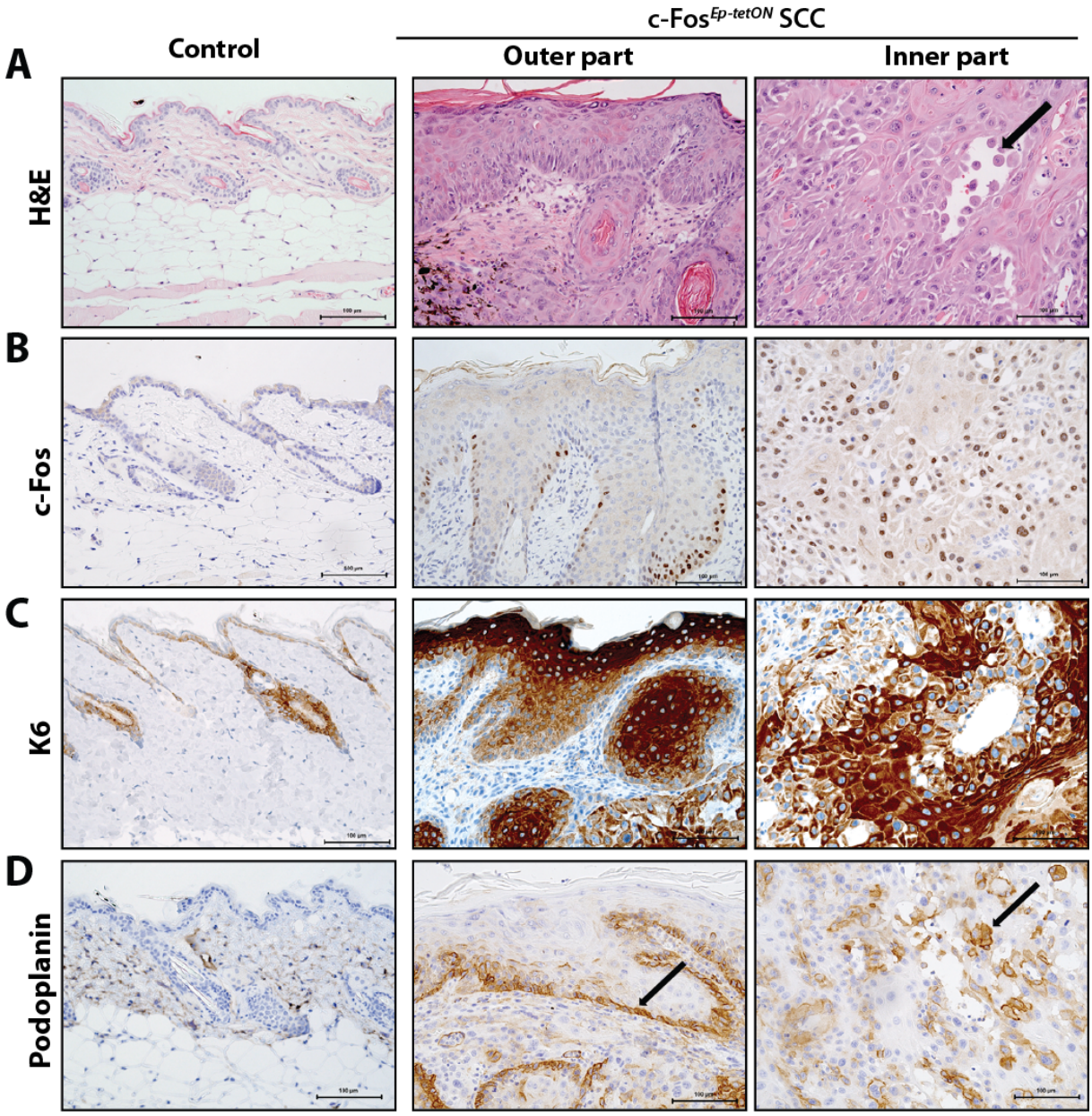
Histologically SCCs invaded the adjacent dermal and fat layers and presented features of SCCs, such as aberrant mitosis, cell and nuclear atypia, prominent intercellular bridges and keratin pearls (Fig. 21A). IHC analyses revealed that expression of c-Fos was present in the tumor (Fig. 21B). Furthermore, the tumors expressed levels of high keratin 6, a keratin expressed by proliferating keratinocytes (Fig. 21C). In addition, these SCCs also expressed important markers associated with cancer cell migration and invasiveness, such as podoplanin, a direct c-Fos target gene (Durchdewald et al., 2008), which was upregulated in the invading front of the c-Fos$^{Ep\text{-}tetON}$ SCC (Fig. 21D).

Figure 20.



(A) Experimental set-up. 0.5% DMBA diluted in acetone is applied in the back skin of c-Fos$^{Ep\text{-}tetON}$ mice at 5-7 weeks of age. One week after, Dox treatment starts either in the drinking water (2.5g/l) or in the food pellets for 11 weeks. TPA is applied at the same time as Dox in certain mice. (B) Picture of control and c-Fos$^{Ep\text{-}TetON}$ female mice after DMBA and 11 weeks of Dox administration. (C) Representation of the number of lesions per mouse in control and c-Fos$^{Ep\text{-}tetON}$ mice after DMBA and 8 weeks of Dox administration. Mean ±SD of all the lesions developed in males (n=3) and females (n=3). nd= not detected. No lesions found in control mice. (D) Representation of the lesion size in c-Fos$^{Ep\text{-}tetON}$ female (n=3/3) and male (n=3/3) mice after DMBA and 11 weeks of Dox. Mean ±SEM. (E) H&E staining of normal skin from control mouse and SCC in c-Fos$^{Ep\text{-}tetON}$ female mouse after DMBA application and 11 weeks of Dox administration

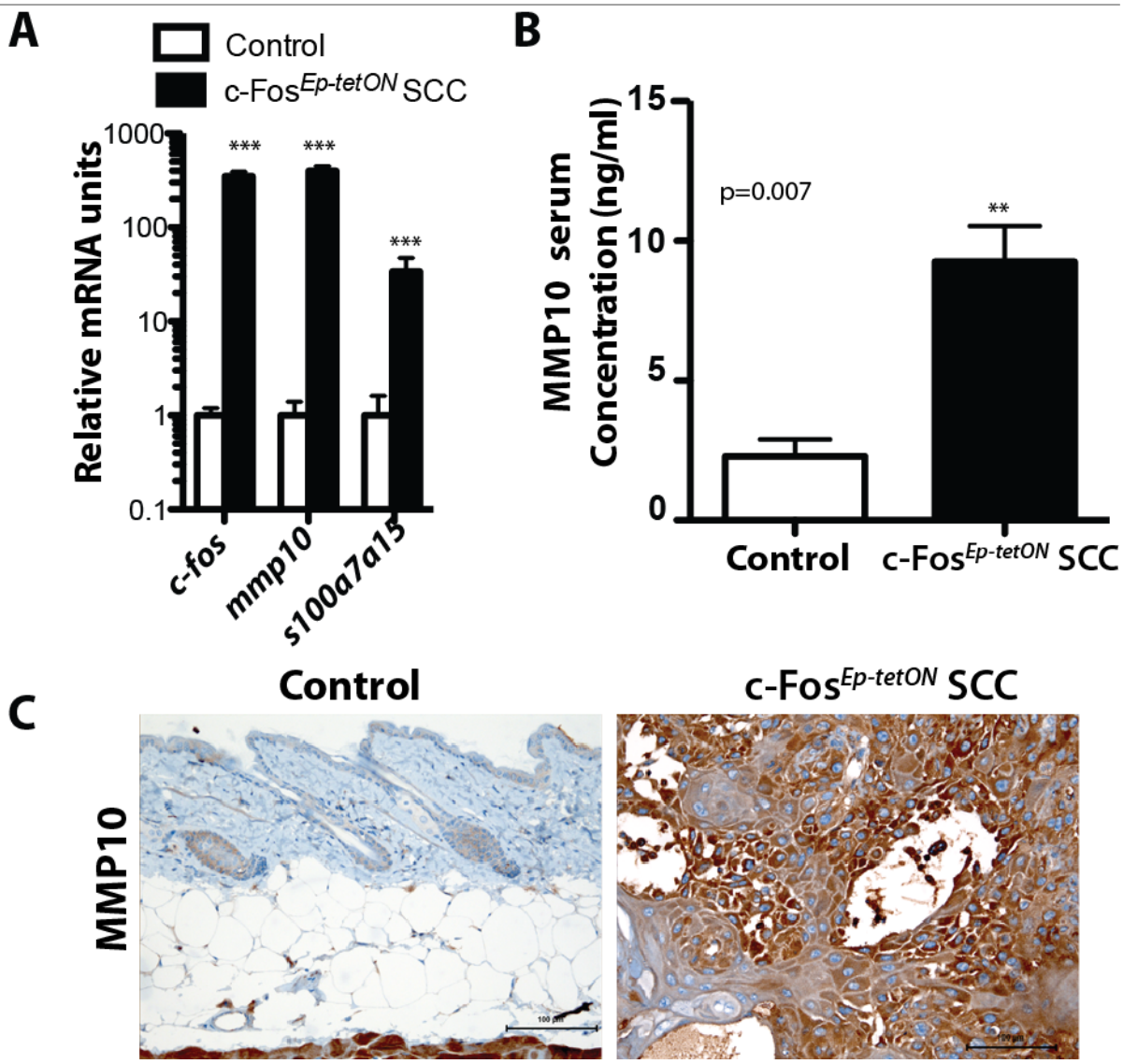
Figure 21.



(A) H&E staining in papilloma from control mouse and SCC in c-Fos$^{Ep\text{-}tetON}$ mouse after DMBA application and 11 weeks of Dox and TPA administration. (n=3/3). (B) Immunohistochemical analyses of c-Fos in the back skin from control mouse and SCC in c-Fos$^{Ep\text{-}tetON}$ mouse after DMBA application and 11 weeks of Dox. (n=3/3). (C) Immunohistochemical analyses of keratin 6 in the back skin from control mouse and SCC in c-Fos$^{Ep\text{-}tetON}$ mouse after DMBA application and 11 weeks of Dox. (n=3/3). (D) Immunohistochemical analyses of Podoplanin in the back skin from control mouse and SCC in c-Fos$^{Ep\text{-}tetON}$ mouse after DMBA application and 11 weeks of Dox. Arrows indicate invading front of the tumor. (n=3/3).

Expression analyses of the whole tumor revealed increased mRNA levels of *mmp10* as well as *s100a7a15* compared to control skin (Fig.22A). Furthermore, MMP10 protein levels were increased in the sera and in the SCCs of c-Fos$^{Ep\text{-}tetON}$ mice, as assessed by ELISA and by immunostaining respectively (Fig. 22B, 22C). Moreover, immunofluorescence stainings revealed increased levels of S100a7a15 in the epidermis of c-Fos$^{Ep\text{-}tetON}$ mice 4 weeks after inducible expression of *c-fos*.

Figure 22.



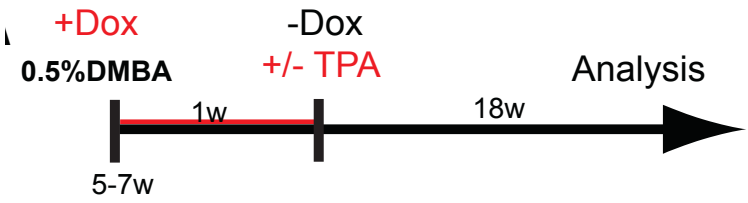
(A) RT-qPCR analyses of *c-fos, mmp10* and *s100a7a15* expression of back skin of control and SCC of c-Fos$^{Ep\text{-}tetON}$ mice. (n=4). (B) MMP10 levels in the serum of control and c-Fos$^{Ep\text{-}tetON}$ mice bearing an SCC. (n=3).(C) Immunohistochemical analyses of MMP10 of the back skin of control and SCC of c-Fos$^{Ep\text{-}tetON}$ mice. (n=3). **p<0.005. ***p<0.005.

7.2. c-Fos$^{Ep\text{-}tetOFF}$ mice develop highly invasive SCCs upon DMBA

To confirm our finding suggesting that c-Fos acts as a tumor promoter in the development of SCCs, we carried out the same adapted version of the DMBA/TPA skin carcinogenesis protocol to the c-Fos$^{Ep\text{-}tetOFF}$ mouse line (Fig. 23).

Control and c-Fos$^{Ep\text{-}tetOFF}$ mice were subjected to a single dose of 0.5% DMBA topically applied to the back skin of these mice (at 5-6 weeks of age) and one week later Dox was removed to induce *c-fos* expression for 18 weeks. c-Fos$^{Ep\text{-}tetOFF}$ mice developed invasive SCCs. These results confirm the results obtained by using the c-Fos$^{Ep\text{-}tetON}$ mouse line. However, the development of the SCCs occurred with a 7-week difference compared to the c-Fos$^{Ep\text{-}tetON}$ mouse line, possibly due to differences in the K5 transgenic mouse line or *c-fos* induction levels.

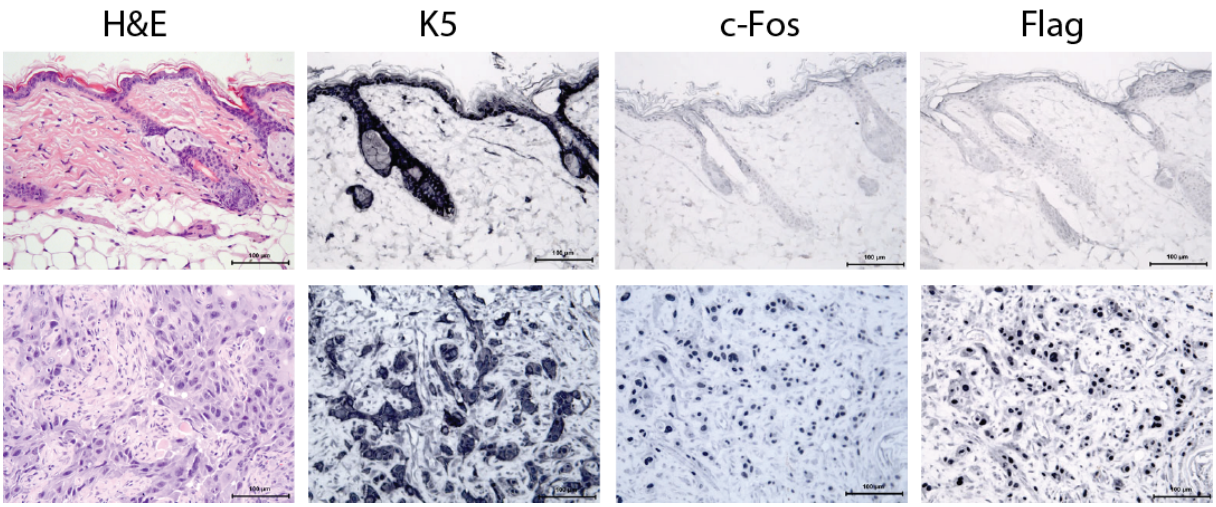
Figure 23.



Experimental set-up. Adapted skin carcinogenesis protocol. 0.5% DMBA diluted in acetone is applied in the back skin of c-Fos$^{Ep\text{-}tetOFF}$ mice at 5-7 weeks of age. One week after, Dox treatment is removed from the food. Mice are killed and analyzed 18 weeks later. (n=11/13).

Histological analyses of back skin and SCCs revealed highly invasive SCCs, as seen by H&E. These SCCs expressed c-Fos protein as well as markers expressed by invasive and proliferative keratinocytes such as podoplanin and K6. Moreover, islets of K5-expressing keratinocytes were observed invading the neighboring dermis (Fig. 24). These data indicate that sustained increased levels of c-Fos in the epidermis promote epidermal carcinogenesis after DMBA treatment.

Figure 24.



Immunohistochemical analyses (H&E, Keratin 5 (K5), c-Fos, Flag) of back skin of control and SCC c-Fos$^{Ep\text{-}tetOFF}$ mice treated with 0.5% DMBA and 18 weeks off. (n=3/3).

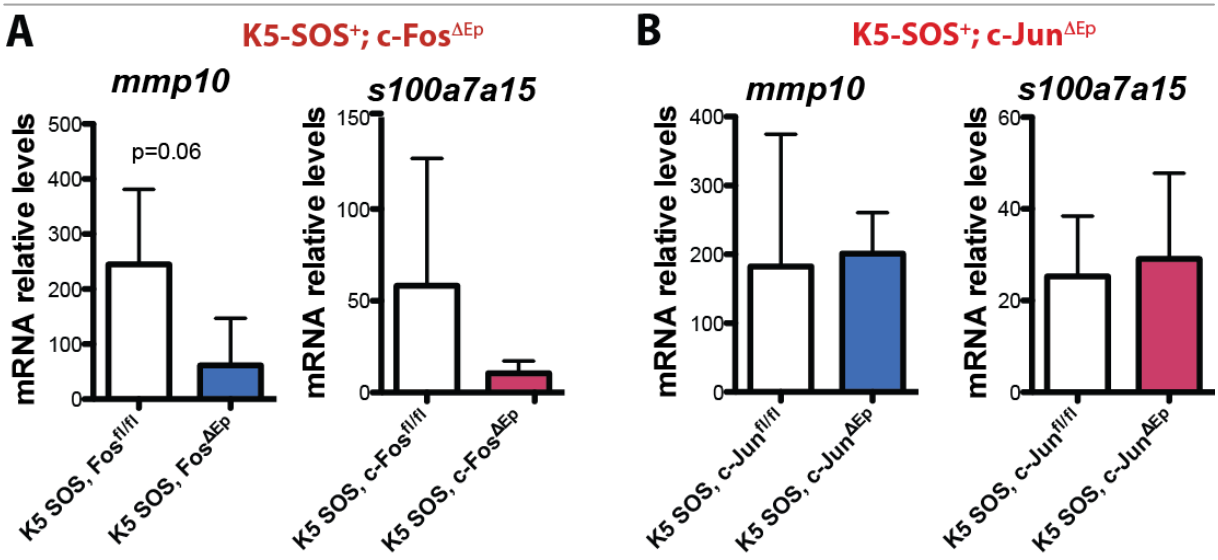
7.3. Impaired *mmp10* and *s100a7a15* expression in *c-fos* deficient K5-SOS^{+} tumor-prone mice

Previous work from our group revealed an important role of c-Fos in the development of skin tumors (Guinea-Viniegra et al., 2012). In these experiments, the K5-SOS^{+} papilloma-prone mouse model, which develop big papillomas in the tail skin due to a hyperactivation of the Ras signaling pathway, was used (Sibilia et al., 2000). In epidermis-specific *c-fos*-deficient K5-SOS^{+} mice (K5-SOS^{+}; c-Fos$^{\Delta Ep}$), tumor development is suppressed due to a hyperactivation of the p53/TACE-dependent Notch signaling pathway that promotes irreversible keratinocyte differentiation leading to tumor suppression.

We employed this mouse model (K5-SOS^{+}; c-Fos$^{\Delta Ep}$) to assess the levels of the two novel targets (MMP10 and S100a7a15). Expression of *mmp10* and *s100a7a15* was decreased in K5-SOS^{+}; c-Fos$^{\Delta Ep}$ mice compared to K5-SOS^{+};c-Fos$^{fl/fl}$ papillomas (Fig, 25A). As a control, we employed another mouse model, the K5-SOS^{+}; c-Jun$^{\Delta Ep}$. These mice also develop smaller papillomas in the absence of c-Jun, another well-known AP-1 family member (Zenz et al., 2003). Therefore, we decided to assess whether the levels of expression of *mmp10* and *s100a7a15* are altered in the absence of c-Jun. However, the levels of these two targets remained intact, suggesting that c-Jun does not

transcriptionally control the expression of these two novel target genes (Fig, 25B).

Figure 25.



(A) RT-qPCR analyses of *mmp10* and *s100a7a15* expression in tumor-prone K5-SOS^{+}; c-Fos$^{fl/fl}$ mice and in K5-SOS^{+}; c-Fos$^{\Delta Ep}$ mice at 4 weeks of age. (n=3/3). (B) RT-qPCR analyses of *mmp10* and *s100a7a15* expression in tumor-prone K5-SOS^{+}; c-Jun$^{fl/fl}$ mice and in K5-SOS^{+}; c-Jun$^{\Delta Ep}$ mice at 4 weeks of age. (n=3/3).

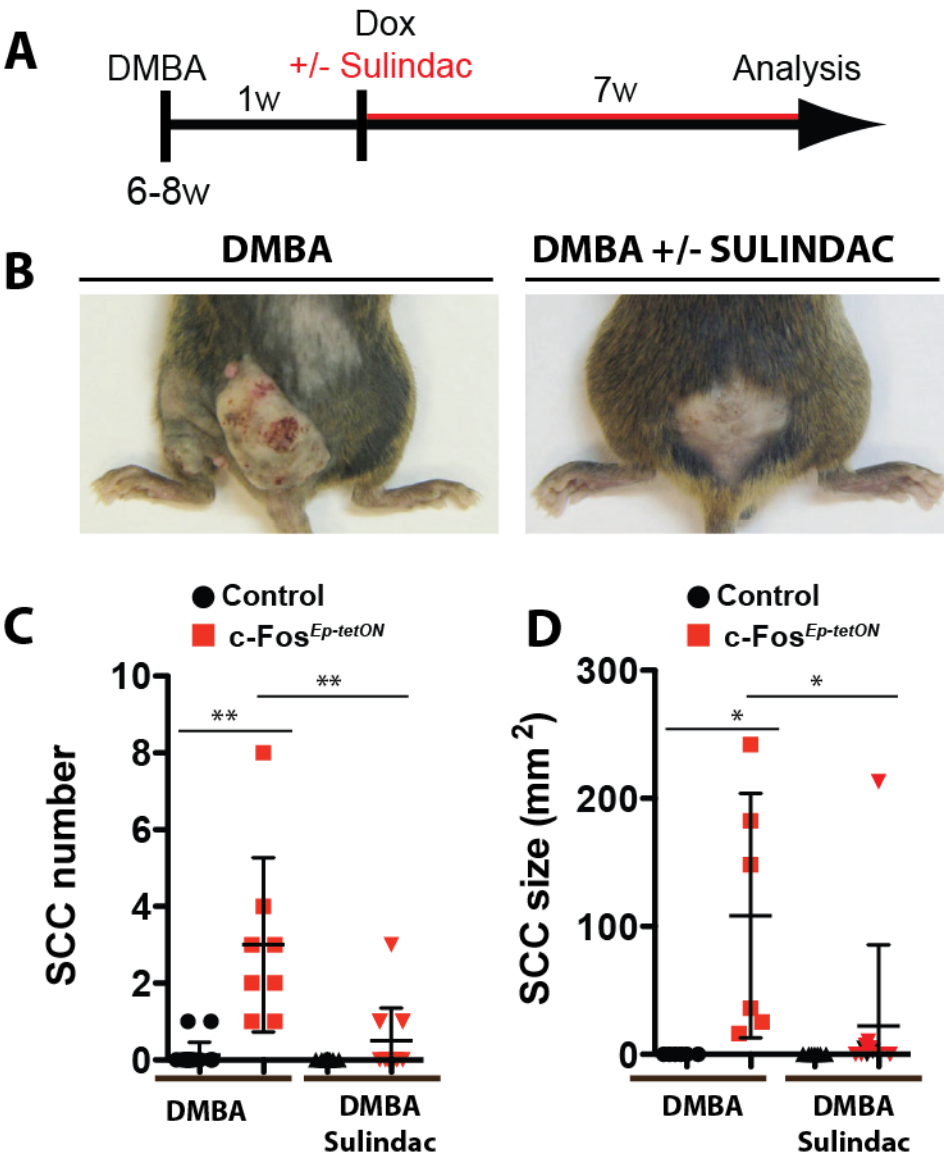
7.4. Sulindac treatment reduces SCC size and number

To test the hypothesis that c-Fos indirectly promotes epidermal proliferation and subsequently SCC development due to the recruitment of inflammatory cells, specifically CD4 T cells, we aimed to inhibit inflammation by using Sulindac. This Non-Steroidal-Anti-Inflammatory-Drug (NSAID) specifically blocks COX1/2 activity and consequently impairs prostaglandin synthesis and inflammatory processes. Sulindac has been widely used to treat patients with inflammatory diseases, such as rheumatoid arthritis and ankylosing spondylitis as well as it is being used in phase II clinical trials for lung cancer chemoprevention (Limburg et al., 2012).

We performed our adapted version of the 2-step carcinogenesis protocol comparing sulindac-treated vs sulindac-untreated c-Fos$^{Ep\text{-}tetON}$ DMBA-treated mice (Fig. 26A.). After one dose of topical DMBA and 7 weeks of inducible *c-fos* expression, mutant mice developed skin tumors (Fig. 26B). Interestingly, Sulindac-treated mice developed fewer tumors and the ones developed were significantly smaller (Fig. 26C and 26D). We

therefore conclude that c-Fos is sufficient to promote SCC development by promoting a microenvironment infiltrated by inflammatory cells and that prevention of inflammatory responses diminishes SCC incidence in c-Fos$^{Ep\text{-}tetON}$ mice.

Figure 26.



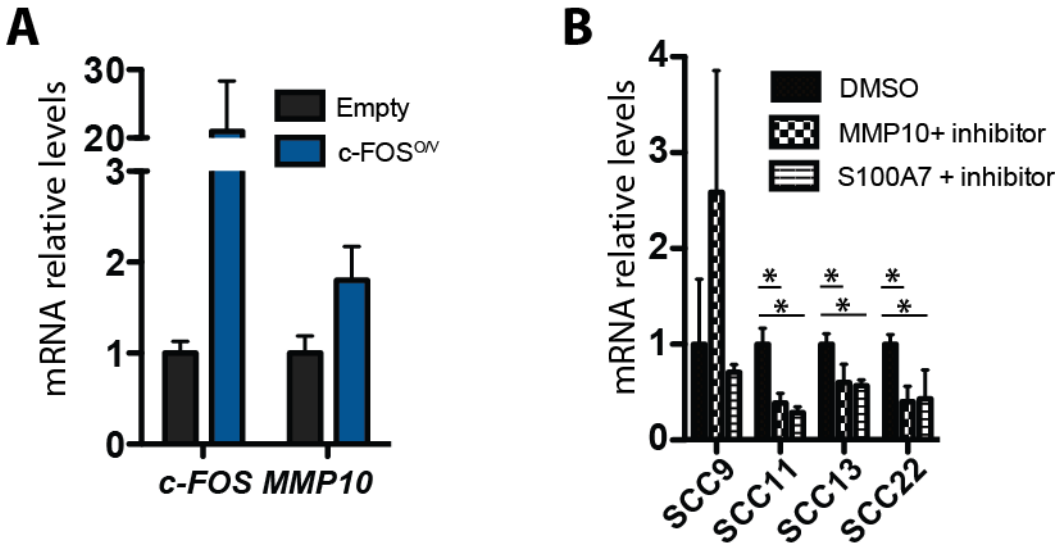
(A) Experimental set-up. 0.5% DMBA diluted in acetone is applied in the back skin of c-Fos$^{Ep\text{-}tetON}$ mice at 6-8 weeks of age. One week after, Dox treatment starts in the food and sulindac is supplied in the drinking water at a concentration of 180mg/L. 7 weeks after mice are sacrificed and analyzed. (n=16/8/19/14). (B) Picture of c-Fos$^{Ep\text{-}tetON}$ mice after DMBA and 7 weeks of Dox ±sulindac. (C) Representation of the number of lesions per mouse in control and c-Fos$^{Ep\text{-}tetON}$ mice after DMBA and 7 weeks of Dox administration. Mean ±SD. (n=16/8/19/14). (D) Representation of the number of lesions per mouse in control and c-Fos$^{Ep\text{-}tetON}$ mice after DMBA and 7 weeks of Dox administration. Mean ±SD. (n=16/8/19/14). *p<0.05, **p<0.005.

8. HUMAN SCCS EXPRESS HIGH C-FOS PROTEIN LEVELS CORRELATING WITH HIGH MMP10 BUT NOT WITH S100A7 EXPRESSION LEVELS

In order to address whether the findings observed in the c-Fos$^{Ep\text{-}tetON}$ and c-Fos$^{Ep\text{-}tetOFF}$ mouse models were applicable to human, we tested mRNA expression levels of MMP-10 and S100A7 by RT-qPCR using human epidermal primary keratinocytes (HEKs) that overexpressed c-FOS. These HEKs had been previously infected with lentiviruses containing either empty vector or *c-FOS* over-expressing vector under the control of the ubiquitous CMV promoter. Upon *c-FOS* expression, we observed an upregulation of *MMP-10* mRNA levels (Fig. 27A). However, no correlation was observed with *S100A7* (data not shown). These data confirm our results obtained using mouse models and suggest *MMP10* as a c-FOS target gene in human. However, ChIP analyses will be performed to assess binding of c-FOS to the human *MMP10* promoter.

Furthermore, RT-qPCR analyses were performed in human SCC cell lines to address the functional relevance of c-*FOS* upregulation in human SCCs and the correlation with *S100A7* and *MMP10* expression. We used 4 highly expressing *c-FOS* SCC cell lines. Upon c-FOS/AP-1 inhibitory treatment (Aikawa et al., 2008), we observed that in 3 out of 4 cell lines, *MMP10* as well as *S100A7* levels were also reduced (Fig. 27B). in human there are two genes and consequently two proteins (S100A7 and S100A15) compared to mouse, which only has one *s100a715* gene (Wolf et al., 2011). S100A7 and S100A15 are highly homologous, but distinct in regulation and function, and are exclusively expressed. We therefore conclude c-FOS likely directly controls the expression of *MMP10* and *S100A7.* However, further analyses will be performed to assess the mRNA levels of S100A15 by using specific primers provided by Ronald Wolf and compare them to the levels of S100A7. Finally, ChIP analyses should be performed to test binding of c-FOS to the promoters of *MMP10* and *S100A7* and *S100A15.*

Figure 27.



(A) RT-qPCR analyses of *c-FOS* and *MMP10* Human Epidermal keratinocytes infected with lentiviruses containing empty vector or with a vector to overexpress c-FOS (c-FOS O/V). (B) RT-qPCR analyses of *MMP10* and *S100A7* Squamous Cell Carcinoma Cell lines treated with c-FOS/AP-1 inhibitor or with vehicle (DMSO).

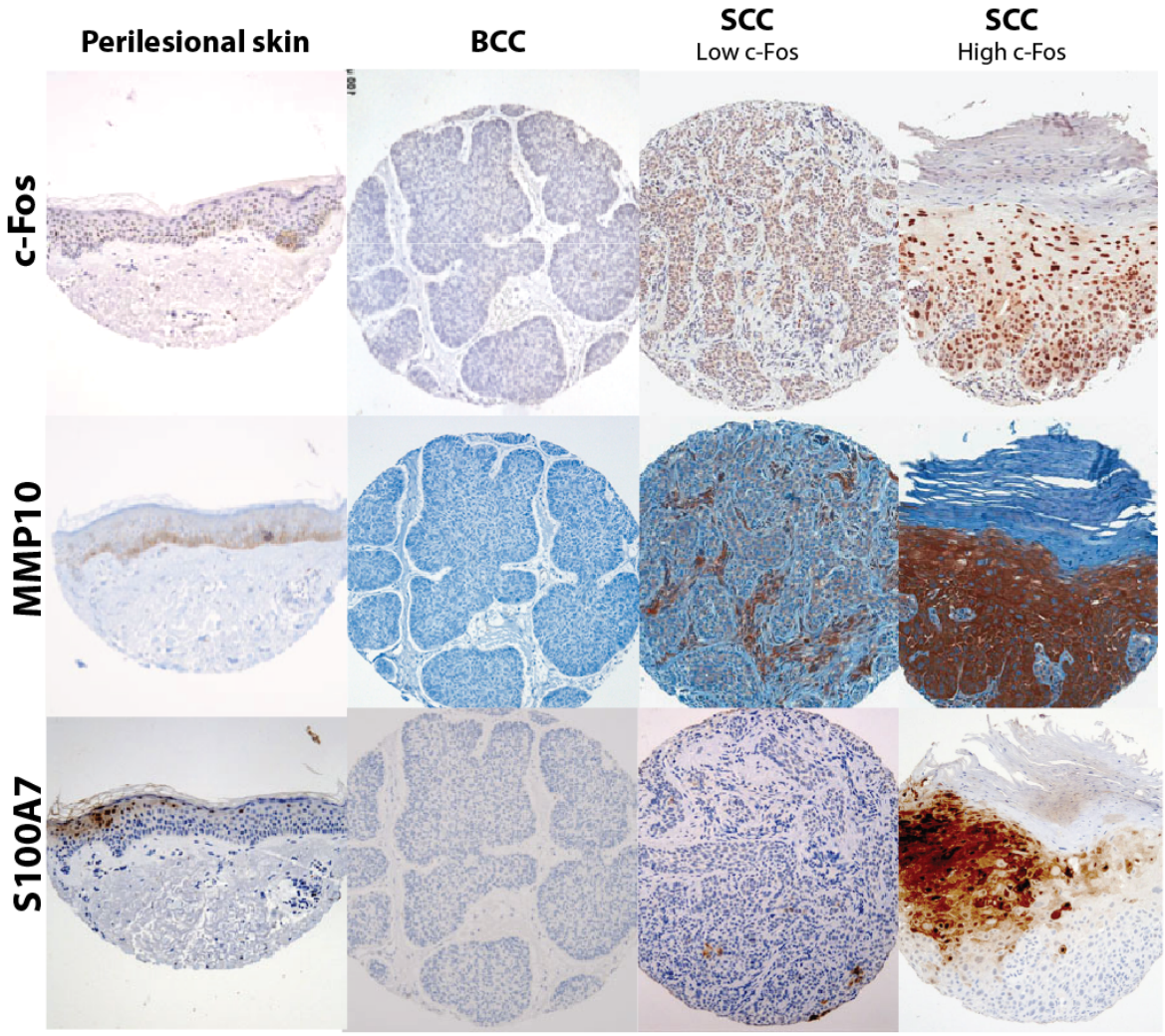
8.1. SCCs but not BCCs express c-Fos and this correlates with MMP10 expression

To determine the relevance of our findings to human Non-Melanoma Skin Cancer (NMSC), in collaboration with Peter Petzelbauer (Medical University of Vienna, Austria), we examined c-FOS, MMP10 and S100A7 expression in human epidermal tumors. Sections from 85 basal cell carcinomas (BCC) and 96 SCCs patients, as well as 20 perilesional skin samples, were analyzed by IHC (Figure 24A).

In BCCs, none of the samples expressed c-FOS protein, correlating with no MMP10 expression (Fig. 28A). In contrast, 75% of SCC samples expressed high c-FOS protein levels, perfectly correlating with MMP10 expression. 25% percent of the SCCs that expressed low c-FOS levels correlated with low or inexistent MMP10 levels (Fig. 28B). However, we found no correlation between c-FOS and S100A7 protein levels in the same set of samples. R. Wolf *et al* previously described that in human there are two genes and consequently two proteins (S100A7 and S100A15) compared to mouse, which only has one *s100a715* gene (Wolf et al., 2011). S100A7 and S100A15 are highly homologous, but distinct in regulation and function, and are exclusively expressed.

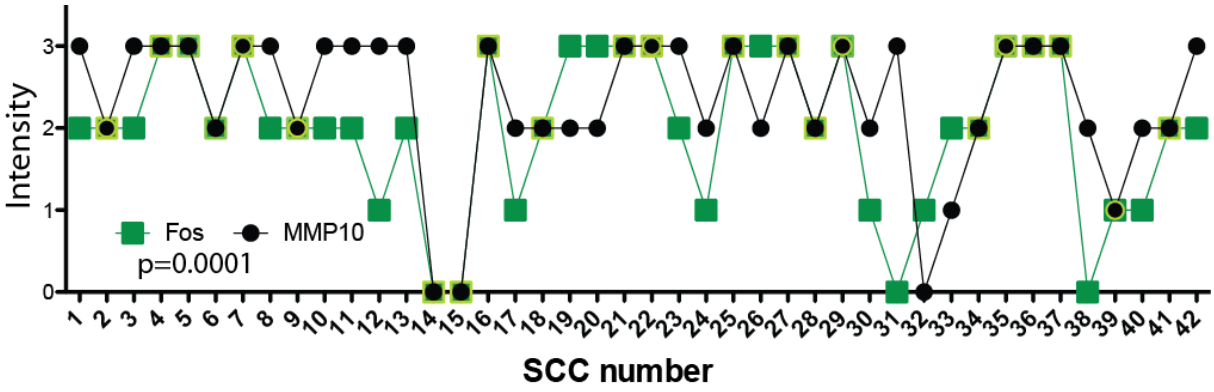
S100A7 mainly expressed in differentiated cells within the epidermis and S100A15 expressed in basal keratinocytes. We are currently performing immunostainings for S100A15 to evaluate whether c-Fos correlates with S100A15 expression. These findings suggest MMP10 is likely a direct transcriptional target of c-FOS in human and both proteins could be useful targets to treat SCCs of the skin by means of inhibitory molecules as a preventive approach.

Figure 28.



Immunohistochemical analyses of c-FOS, MMP10 and S100A7 in human perilesional skin, BCC, SCC (low and high levels of c-Fos). (”perilesional skin” n=10, BCC=216, SCC=96).

Figure 29.



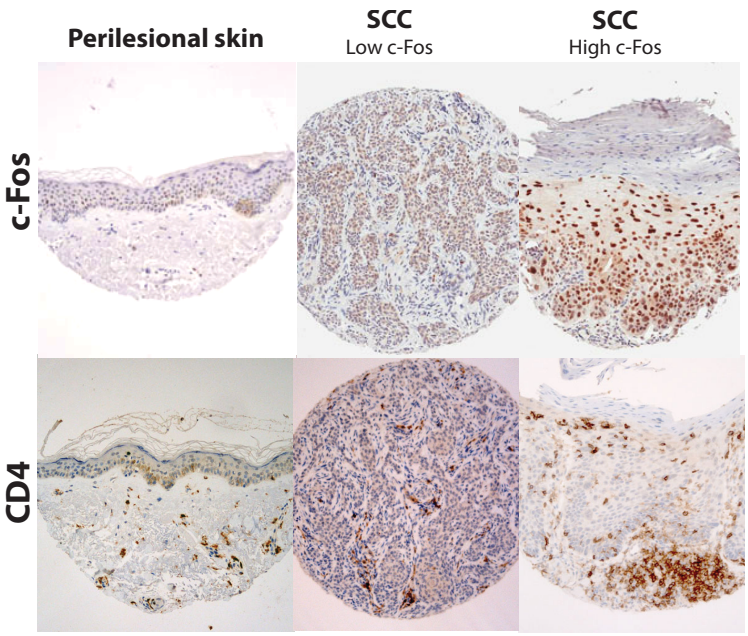
Graph depicting the correlation between c-FOS and MMP10 staining in human SCCs. (n=42).

8.2. c-FOS protein-expressing SCCs present CD4 T lymphocyte infiltrates

We have shown that c-Fos transcriptionally controls S100a7a15 and MMP10 expression in mice and we have also observed a strong correlation between c-FOS and MMP10 expression in human SCCs. Moreover, S100a7a15 has been shown to be involved in the recruitment of CD4 T cells and granulocytes (Wolf et al., 2008) and MMP10 can also facilitate the recruitment of leukocytes from the bloodstream by degrading components of the extracellular matrix and by modulating cytokine and chemokine activity (Elkington et al., 2005). Therefore, we evaluated the levels of CD4 T cell infiltrates by performing immunostainings of CD4 T cells in human SCCs and BCCs.

The levels of CD4 T cell infiltrates were assessed by immunostaining on human samples. Interestingly, 73% of those c-FOS-expressing SCCs showed CD4 T cell infiltrates (Fig. 30). Moreover, since CD4 is also a marker for dendritic cells in the skin, we also tested the levels of CD3 in the same samples and observed similar patterns of expression as CD4 (data not shown), suggesting these CD4 positive cells are likely to be mainly CD4 T Lymphocytes and not dendritic cells. However, we plan to perform co-stainings of both, CD3 and CD4 markers. These data show a strong correlation between c-FOS expression in human SCCs with the levels of CD4 T cell infiltrates. Therefore we propose that targeting chronic CD4 T cell infiltrates could be of therapeutic interest to treating SCCs by using anti-inflammatory compounds.

Figure 30.



Immunohistochemical analyses of c-FOS and CD4 T cells in human perilesional skin and SCC (low and high levels of c-Fos). (”perilesional skin” n=10, SCC=96).

Discussion

1. c-FOS IN EPIDERMAL HOMEOSTASIS

Many of the insights regarding specific functions of c-Fos protein in development and disease have been obtained from genetically modified mice and cells derived thereof. c-Fos is dispensable for skin homeostasis, since global or keratinocyte-specific deletion of *c-fos* does not reveal a skin phenotype (Wang et al., 1992; Guinea-Viniegra et al., 2012). However, under stress conditions, such as UV-light, Ras/SOS activating mutations, TPA or calcium, *c-fos* is up-regulated and phosphorylated. Guinea-Viniegra et *al* demonstrated an active role of c-Fos in mediating stress responses and showed that epidermal *c-fos* deletion in mouse tumor models or pharmacological FOS/AP-1 inhibition in human SCC cell lines induced epidermal cell differentiation and skin tumor suppression by inducing p53 and subsequently TACE (TNFα converting enzyme) expression. TACE, in turn, promotes keratinocyte differentiation by activating the Notch signaling pathway (Guinea-Viniegra et al., 2012). Additionally, c-Fos-phoshorylation knock-in mice, in which c-Fos phosphorylation sites have been substituted by non-phosphorylatable alanines, show reduced papilloma formation in a papilloma-prone backgroud (K5-SOS) (Bakiri et al., 2011). In contrast, overexpression of the viral homolog of c-Fos (v-Fos) in the spinous layer leads to long latency, wound-associated epidermal hyperplasia (Greenhalgh et al., 1995). Altogether, these data indicate that c-Fos is necessary for efficient papilloma formation.

I have generated two mouse models to inducibly express *c-fos* under the control of the keratin 5 promoter, which is expressed in basal keratinocytes as well as in the basal layer of other stratified epithelia, to assess specific functions of c-Fos in the epidermis. Expressing *c-fos* in the basal layer of the epidermis leads to a disruption of the epidermal architecture with the development of preneoplastic lesions by four or sixteen weeks of inducible *c-fos* expression in c-Fos$^{Ep\text{-}tetON}$ and c-Fos$^{Ep\text{-}tetOFF}$ mice, respectively. These lesions most likely did not fully convert into SCCs due to the early death of these mice before the tumors developed. Moreover, other features such as hair loss, hyperkeratosis (thickening of the *stratum corneum*), parakeratosis (retention of nuclei in the *stratum corneum*) as well as follicular cysts (follicles in which there is proliferation and accumulation of keratin into a cystic mass that invaginates into the dermis) were observed. The features observed in this mouse model are often present in

certain inflammatory skin diseases such as Psoriasis, Atopic Dermatitis and Actinic Keratosis (AK) (Bovenschen et al., 2005; Berhane et al., 2002), which are described as preneoplastic lesions and these lesions often develop to SCCs (Mortier et al., 2002).

The disruption of epidermal homeostasis promoting hyperplasia can happen as a consequence of an imbalance in different cellular processes, proliferation and differentiation, or can also develop as a consequence of the epithelial barrier disruption (Hsu and Fuchs, 2012). Upon inducible *c-fos* expression *in vivo*, increased proliferation was observed by performing Ki67 immunostainings. Interestingly, no cell autonomous changes in proliferation were observed upon *c-fos* expression in primary c-Fos^{tetON} keratinocytes, as assessed by three independent methods. Thus, these data suggest that increased proliferation *in vivo* must occur from changes arising in the stroma of the preneoplastic lesion, probably due to paracrine signaling of growth factors or cytokines from stromal cells. These results are in line with previous findings showing that mice lacking epidermal c-Fos do not develop any alteration in proliferation neither *in vivo* nor *in vitro* (Guinea-Viniegra et al., 2012). However, the impact of *c-fos* expression on cell proliferation is cell-type-specific, since it can promote cell cycle progression in osteoblasts and in mouse embryonic fibroblasts (MEFs) by activating the expression of Cyclins D1, A and E (Sunters et al., 1998).

Furthermore, epidermal differentiation was assessed *in vivo* by performing immunostainings of early and late differentiation markers of backskin of c-Fos$^{Ep\text{-}tetON}$ mice and controls after inducible expression of *c-fos.* A delay in keratinocyte differentiation was clearly observed, since basal keratinocytes expanded from one to several layers of keratinocytes. Interestingly, a similar defect was observed when c-Fos^{tetON} keratinocytes were primed to differentiate *in vitro* upon Ca^{2+} treatment, where keratinocytes expressing inducible *c-fos* failed to properly differentiate. mRNA expression analyses revealed impaired expression of early and late differentiation markers as well as impaired activation of the differentiation-promoting p53/TACE/Notch-signaling pathway. These findings are in line with previously published data showing that a reduction in c-Fos levels in keratinocytes or *c-fos*-deficient keratinocytes undergo premature differentiation upon certain stress responses, such as Ca^{2+} and TPA in a p53 and TACE dependent manner (Guinea-Viniegra

et al., 2012). Therefore, and since keratinocyte differentiation is impaired upon c-Fos expression *in vitro,* I evaluated the integrity of the epidermal barrier, which can occur as a consequence of impaired differentiation due to reduced Notch signaling (Demehri et al., 2009). No epidermal barrier defects were observed upon c-Fos expression in c-Fos$^{Ep\text{-}tetOFF}$ pups or in back skin of c-Fos$^{Ep\text{-}tetON}$ mice by performing inside-out and outside-in epidermal barrier permeability tests. These results exclude the possibility of the barrier defect being a direct cause promoting keratinocyte proliferation and the subsequent development of the "preneoplastic lesions" even though Notch signaling is reduced.

Tet-ON and tet-OFF mouse models allow spatiotemporal modulation of inducible gene expression. I exploited this feature to address the question whether the observed preneoplastic lesions are dependent on constitutive *c-fos* expression and whether upon cessation of *c-fos* expression, the lesions would regress. I analyzed the "reverted" mice after switching off *c-fos* expression once the lesions had already developed. I observed an improvement in the back skin of these mice. Overall, no epidermal hyperplasia was observed even though some follicular cysts still remained. This suggests that hair follicles, once they loose their fate, cannot regenerate their structure thus leading to hair loss. Moreover, some isolated patches still presented mild hyperplasia and these areas coincided with still enhanced expression of *c-fos*. Overall, these findings suggest that the preneoplastic lesion requires constant *c-fos* signaling to maintain these pathological features.

2. c-FOS TRIGGERS INFLAMMATORY PROCESSES IN THE SKIN THAT LEAD TO THE DEVELOPMENT OF PRENEOPLASTIC LESIONS

Immune responses in the skin are a crucial component of the peripheral immune system to protect against pathogens to obtain information for the immune system to initiate specific and long-term immunity and finally to repair damaged tissue. Acute inflammation is a rapid self-limiting process, however, it does not always resolve quickly and may be maintained for a prolonged time and become chronic due to constant infection. There is increasing evidence that chronic inflammation is at the basis of many of the diseases of advanced age, such as cancer (Berhane et al., 2002).

Here, I have described a new role of c-Fos in promoting immune-cell-mediated preneoplastic lesions in the epidermis. Flow Cytometry analyses of back skin revealed a transient granulocyte and chronic $CD4^{+}$T cell recruitment following inducible *c-fos* expression. These infiltrates were present in the skin of c-Fos$^{Ep\text{-}tetON}$ mice compared to controls after inducible *c-fos* expression, when no visible lesions in the skin had developed, indicating that, recruitment of these cells preceded the development of focal preneoplastic lesions. Interestingly, the AP-1 family of transcription factors (mainly c-Jun, JunB and Fra2) has also been previously involved in mediating skin inflammatory processes by inhibiting the expression of cytokines such as G-CSF, IL-6, IL-17, TNFα or TSLP (Zenz et al., 2005; Meixner et al., 2008; Guinea-Viniegra et al., 2009; Schonthaler et al., 2009) (Wurm and Wagner, unpublished).

Using FACS (Fluorescent-activated cell sorting) sorting followed by mRNA expression analyses, I identified Interleukin-22 (IL-22) as the main cytokine expressed by CD4 T cells that infiltrate c-Fos$^{Ep\text{-}tetON}$ preneoplastic lesions. IL-22 is a member of the IL-10 family, which includes IL-19, IL-20, IL-24 and IL-26. This cytokine is mainly expressed by Th17 cells at barrier surfaces (Liang et al., 2006). IL-22 expression is restricted to cells of lineages of the innate and adaptive immune responses, whereas expression of the functional IL-22R seems to be restricted to the non-hematopoietic cells of the skin, pancreas, intestine, liver, lung and kidney (Tachiiri et al., 2003; Wolk et al., 2004). IL-22-IL-22R interactions can induce the expression of genes encoding molecules involved in tissue inflammation, immunesurveillance and homeostasis (Liang et al., 2006). One of the signaling pathways activated upon IL-22 IL-22R ligation is the JAK-STAT3 pathway, which is involved in keratinocyte survival and proliferation (Zhang et al., 2012). Consistent with this, deregulated expression of IL-22 or IL-22R has been reported in certain human diseases, such as psoriasis (Pan et al., 2012), and studies in mouse models have identified crucial roles for IL-22–IL-22R in regulating homeostasis of epithelial cells at barrier surfaces. I propose here an important contribution of CD4 T cell secreted IL-22 in promoting keratinocyte survival and proliferation thus priming the keratinocytes towards uncontrolled proliferation that may eventually lead to cancer.

Interestingly, activation of STAT3 and NF-κB pathways was observed upon inducible *c-fos* expression in c-Fos$^{Ep\text{-}tetON}$ mice. Both pathways are usually not expressed

during epidermal homeostasis, however these are activated upon inflammatory processes and cancer (You et al., 2012). The activation of STAT3 pathway is likely a consequence of IL-22 signaling from the infiltrating CD4 T lymphocytes.

To ask whether the preneoplastic lesions observed upon *c-fos* expression require the presence of CD4 T cells, mature T and B cells were depleted by crossing the c-Fos$^{Ep\text{-}tetON}$ mouse model to a Rag1$^{-/-}$ mouse. Interestingly, 80% of the mice in a Rag1-deficient background showed either no lesions or the lesions were significantly smaller. These results clearly point to the relevance of CD4 T cells in the development of skin preneoplastic lesions. This results support previous evidence in which there is reduced incidence of development of SCCs in the skin in K14-HPV skin tumor prone mouse in a Rag1- or CD4 T cell-deficient background was found (Daniel et al., 2003; de Visser and Coussens, 2005).

Moreover, flow Cytometry analyses performed after switching off *c-fos* expression revealed a mild, but not significant increase in the CD4 T cell population in the back skin of c-Fos$^{Ep\text{-}tetON}$ mice compared to controls. These results confirm the studies describing that in inflamed skin, due to UV light exposure or infection, some infiltrated CD4 T cells, remain in the peripheral tissue waiting for a second infection or injury where inflammatory processes are triggered.

c-Fos also contributes to the development of other inflammatory diseases such as arthritis (Shiozawa and Tsumiyama, 2009). Aikawa and colleagues, by using a collagen-induced arthritis mouse model (Brand et al., 2007), showed that treatment with a specific c-Fos/AP-1 inhibitor (T5224) before the onset of arthritis inhibited the development of this inflammatory disease. Interestingly, in 91% of the cases joint destruction, was inhibited in treated mice compared to controls (Aikawa et al., 2008). This inhibitor was also used as a therapeutic treatment once the arthritis had developed. However, this regimen did not cause a substantial improvement in joint destruction, but there was an improvement in the indices of cell infiltration, neutrophil infiltration, and synovial cell proliferation. Mechanistically, the authors identified a pro-inflammatory cytokine IL-1β and MMP-3 to be significantly lowered in the serum of T5224-treated mice, but did not provide further evidence of the cellular source of these molecules (Aikawa et al., 2008).

Depending on the cell context, c-Fos can also exert anti-inflammatory effects. Upon LPS (Lipopolysaccharyde) treatment, complete c-Fos knock-out mice showed increased expression levels of IL-6, TNFα, IL-12p40 in their spleens (Ray et al., 2006). This was due to increased NF-κB levels and activity in the absence of c-Fos in macrophages. In line with these findings, the same group observed that c-Fos knock-out mice were more susceptible to DSS-induced colitis without defining the molecular mechanism underlying this phenotype (Takada et al., 2010). Moreover, Koga and colleagues further demonstrated by silencing *c-fos* expression in dendritic and macrophage cell lines that c-Fos physically interacts with p65 and impairs c-AMP-induced, p65-mediated TNFα expression (Koga et al., 2009). This direct interaction between c-Fos and p65/NF-κB could likely explain the findings observed by Ray and colleagues in which upon c-Fos deficiency in macrophages, NF-κB levels and signaling are increased (Ray et al., 2006).

Overall, it is known that c-Fos can exert pleiotropic roles depending on the cell context. c-Fos knock-out mice show reduced B-cell numbers in the spleen, lymph nodes and peripheral blood due to a marked reduction in the number of B-cell precursors (Okada et al., 1994). Moreover, c-Fos knock-out mice also showed a considerable inhibition of early T cell development with a significant lower thymocyte number compared to wild-type mice (Wang et al., 1992). This phenotype is a consequence of an unexpected function of c-Fos as a direct regulator of T cell antigen recpetor-β (TCRβ) recombination by binding to an AP-1 binding site in a recombination signal sequence and physical interaction with RAG recombinases (Wang et al., 2008). Contrary to c-Fos knock-out mice that display impaired B and T cell development, c-Fos transgenic mice have impaired T cell function. This effect is indirectly mediated by c-Fos expression inducing increased proliferation of thymic epithelial cells, which indirectly affects T cell development (Ruther et al., 1988).

In summary, the transcription factor c-Fos, exerts pleiotropic roles, which are cell- and context-specific. I have shown that c-Fos promotes inflammatory responses when inducibly expressed in the epidermis of c-Fos$^{Ep\text{-}tetON}$ mice even though in other cell types, such as macrophages, upon stimulus, it can inhibit expression of certain cytokines (Ray et al., 2006).

3. c-FOS TRANSCRIPTIONALLY CONTROLS *mmp10* AND *s100a7a15* EXPRESSION

In order to identify novel transcriptional targets of c-Fos in keratinocytes that could provide mechanistic insights in the phenotype developed in c-Fos$^{Ep\text{-}tetON}$ mice, we performed genome-wide expression analyses in collaboration with Peter Angel (DKFZ, Heidelberg). These analyses revealed no major changes in the expression of genes involved in keratinocyte differentiation neither in proliferation upon inducible *c-fos* expression. However, 5 consistently up-regulated target genes were identified (*mmp10, tgfbi, eef2, ngf and s100a7a15)*. Amongst these genes, further characterization of *mmp10* and *s100a7a15* was performed due to their implication in inflammatory responses as well as in skin cancer. I validated the expression of these two candidate genes by mRNA and protein analyses in c-Fos^{tetON} keratinocytes *in vitro* as well as in the back skin of c-Fos$^{Ep\text{-}tetON}$ and control mice induced with Dox. Moreover, Chromatin IP (ChIP) analyses revealed direct binding of c-Fos to the promoters of these genes, indicating that c-Fos transcriptionally regulates expression of these two novel target genes. Furthermore, TPA-treated control and *c-fos*-deficient keratinocytes were used as a control to directly show impaired expression of *mmp10* and *s100a7a15* upon c-Fos deficiency.

MMP10, matrix metalloprotease 10, also known as Stromelysin 2, plays important roles in physiological processes, such as embryonic development and tissue remodeling and cancer (Kessenbrock et al., 2010). The MMP family of proteins comprises 20 human MMP members divided in five main groups according to their structure and substrate specificity: collagenases, gelatinases, membrane type, stromelysins and matrilysins (Bourboulia and Stetler-Stevenson, 2010). Stromelysins cleave similar substrates (collagens III, IV and V, fibronectin, elastin) and also these enzymes cleave other MMPs, which are secreted as inactive enzymes or zymogens, such as proMMP-1, proMMP-7, proMMP-8 and proMMP-9 (Parks et al., 2004). In the epidermis, MMP10 is usually expressed at low levels, but only upon wounding it is upregulated at the tip of the migrating epithelial tongue, similar to what we observe in c-Fos$^{Ep\text{-}tetON}$ mice comparing early and late stages of phenotype development (Saarialho-Kere et al., 1994; Madlener et al., 1996). Moreover, the group of Werner generated a transgenic mouse model constitutively expressing active MMP10 in keratinocytes. These

animals had no alterations in the skin architecture, although upon wounding, keratinocytes at the migrating epidermal tip were scattered and there was reduced deposition of new matrix (Krampert et al., 2004). These results suggest that MMP10 is important in the epidermis during wound healing processes. Interestingly, several MMPs (MMP-1, MMP-3 and MMP-13) have AP-1 binding sites and are regulated by AP-1 family of transcription factors (Tuckermann et al., 2001; Eferl and Wagner, 2003).

I have attempted to inhibit MMP signaling by using a broad MMP inhibitor (TAPI-1) *in vivo* (due to the absence of specific MMP10 inhibitors) supplied systemically to c-Fos$^{Ep\text{-}tetON}$ mice. Preliminary analyses revealed a delay in the development of the phenotype in c-Fos$^{Ep\text{-}tetON}$ mice treated with TAPI compared with mice treated with vehicle. Further experiments will be necessary to prove a clear impact of MMP10 signaling in the recruitment of CD4 T cells and in the development of preneoplastic lesions. Furthermore, other paths of delivery, such as topical administration will be tested.

s100a7a15 gene belongs to the S100 protein family of small calcium-binding proteins involved in epithelial host defense. Whereas in mouse there is only one gene, *s100a7a15*, in human there are two different genes *S100A7* and *S100A15* (Wolf et al., 2006). Genomic analyses of the S100A7 and S100A15 encoding chromosomal regions revealed that both likely evolved by gene duplications during primate evolution, forming an innate subfamily in human (Kulski et al., 2003). The S100A7/A15 proteins are of major interest because of their differential expression and putative functional role in epidermal cell maturation, tumorigenesis and inflammation (Broome et al., 2003) (Eckert et al., 2004). The human calcium-binding protein S100A7 was first identified as a protein upregulated in inflamed hyperplastic psoriatic skin (Madsen et al., 1991). The highly homologous human S100A15 was recently identified in psoriasis and found also to be upregulated in lesional skin (Wolf et al., 2003). Moreover, S100a7a15 induces leukocyte chemotaxis by binding to RAGE (Wolf et al., 2010). Inducible expression of *s100a7a15* in the basal layer of the epidermis predisposes mice to develop "psoriasis-like" phenotype with recruitment of Gr1$^+$ and CD4 T cells (Wolf et al., 2010). In line with these results, genomic and proteomic analyses performed in another psoriasis mouse model (c-Jun/JunB epidermal deletion) also showed increased S100a7a15 mRNA and

protein levels in psoriatic mouse skin (Zenz et al., 2005)(Schönthaler and Wagner, unpublished). Since S100a7a15 is expressed in the preneoplastic lesions developed upon *c-fos* expression and it exerts chemoattractive functions for CD4 T and $Gr1^+$ cells, we plan to inhibit this protein *in vivo* by either using a blocking antibody or by testing an inhibitor, which is specific for S100A9 protein (Kallberg et al., 2012). In conclusion, the contribution of S100a7a15 to the development of preneoplastic lesions is not yet defined.

4. c-FOS FUNCTIONS IN SKIN CANCER DEVELOPMENT AND PROGRESSION

Our studies demonstrate that following DMBA-induced Ha-Ras mutations, c-Fos overexpression in the epidermis is sufficient to promote SCC development in both c-$Fos^{Ep\text{-}tetON}$ and c-$Fos^{Ep\text{-}tetOFF}$ mouse lines. These epithelial tumors were highly proliferative and invaded the surrounding dermis and muscle layer, as assessed by Keratin 6 and Keratin 5 immunostaining.

Our findings confirm previous data indicating that overexpression of the viral homolog of c-Fos (v-Fos) in the spinous layer leads to long latency, wound associated, epidermal hyperplasia (Greenhalgh et al., 1993). In addition, *c-fos* ubiquitous expression (under the control of the H2K promomter) following DMBA-topical applications, lead to the development of SCCs (Sakai, 1990). Conversely, experiments performed with c-Fos knock-out mice have shown that c-Fos is essential for the progression from papillomas to SCCs (Saez et al., 1995). Furthermore, recent data from our lab demonstrate that c-Fos is required for efficient papilloma and SCC formation (Durchdewald et al., 2008; Guinea-Viniegra et al., 2012). In this study, the authors demonstrate that the absence of c-Fos in keratinocyte-specific *c-fos*-deficient mice and upon stress (Ras signaling, calcium or TPA) or in human SCC cell lines treated with the c-Fos/AP-1 inhibitor (T5224) promotes premature keratinocyte differentiation in a p53 and TACE-dependent manner (Guinea-Viniegra et al., 2012).

Mechanistically, c-$Fos^{Ep\text{-}tetON}$ epidermal tumors also expressed both novel c-Fos target genes *mmp10 and s100a7a15* as assessed by mRNA and protein levels. There is increasing evidence that both targets are directly linked to cancer. In mice, genetic

depletion of MMP10 in a KRas-mediated lung cancer model leads to the development of fewer and smaller tumors, and overall decrease in tumor burden compared to controls. However, there are no studies performed in mice implicating an functional role of MMP10 in SCC development. It would be of interest to genetically deplete *mmp10* to address its functional contribution to the development of preneoplastic lesions as well as to the development of SCCs.

In human, increased MMP10 protein levels are expressed in some epithelial tumors such as esophageal or oral SCCs (Liu et al., 2011; Stott-Miller et al., 2011). A strong correlation between MMP10 levels and invasiveness is observed in head and neck tumor samples as well as in cell lines (Deraz et al., 2011). Interestingly, tumoral and salivary MMP10 levels are being used as markers of oral SCCs (Stott-Miller et al., 2011). Moreover, MMP10 is highly expressed in different cancers like lung cancers, oral SCC, esophageal cancer and it has been shown to promote invasion of head and neck cancer (Frederick et al., 2008). In collaboration with Peter Petzelbauer (Medical School University of Vienna, Austria) we found a strong correlation between the expression of c-FOS and MMP10 in SCCs, but not in BCCs. 80% of the SCCs analyzed expressed c-FOS as well as MMP10, but none of the BCCs expressed these two proteins, suggesting that these proteins are important mediators in SCC. These results suggest that c-FOS in human, most likely transcriptionally controls the expression of MMP10 as we have described in mouse. However, chromatin immunoprecipitation analyses to assess binding of c-FOS to the MMP10 promoter should be performed to address this specific question.

there is little evidence linking S100a7a15 protein to cancer. Studies performed in mice, where orthotopic implantation of MVT-1 breast tumor cells into the mammary glands of mice inducibly expressing *s100a7a15* under the control of the Mouse Mammary Tumor Virus (MMTV) promoter, showed enhanced tumor growth and metastasis(Nasser et al., 2012). The authors claim that upon inducible *s100a7a15* expression, ductal hyperplasia occurs and subsequently cytokines and chemoattractant molecules are released to recruit macrophages, which in turn promote tumor development (Nasser et al., 2012). In human there are two genes S100A7 and S100A15, whereas in mice there is only one *s100a7a15.* Several studies have shown upregulation

of S100A7 in several types of cancer, such as breast, laringeal carcinoma, skin SCC, lung SCC, ductal carcinoma and prostate cancer and it has been shown to have a role in promoting endothelial cell proliferation as well as SCC cell migration (Al-Haddad et al., 1999). No studies have been performed so far to address the levels of S100A15 in human cancer. I have used the same set of samples provided by Peter Petezelbauer to meassure the levels of S100A7 protein. Even though increased levels of S100A7 were observed in cutaneous SCCs compared to perilesional skin or to BCCs, c-FOS and S100A7 expression patterns seemed to be exclusive. S100A15 protein levels will be assessed in these samples. These results suggest that there is evidence that S100A7 and S100A15, even though they are very similar in structure, they are different in function.

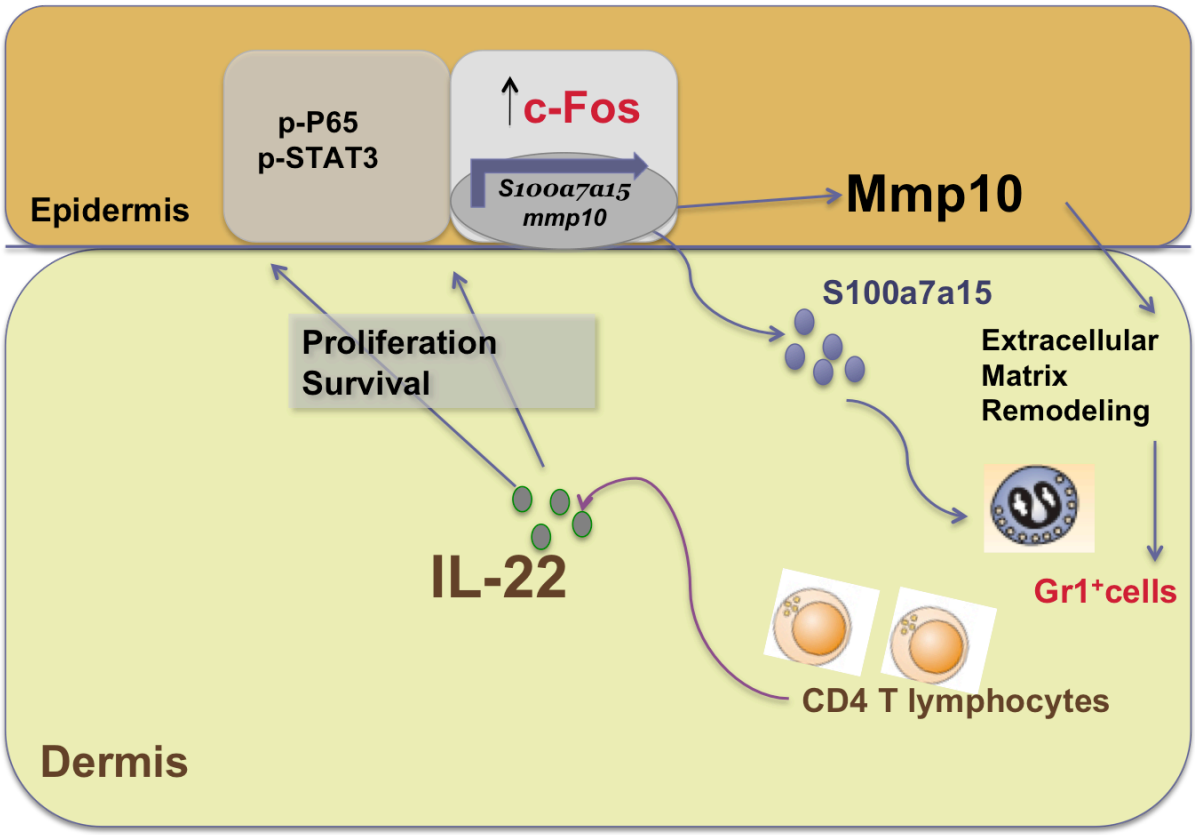
To address the question whether inflammation promotes skin tumorigenesis, I used a Cyclooxygenase 1 and 2 (COX1/COX2) inhibitor (Sulindac). Interestingly, tumor number, size and burden were significantly reduced. COX1 and COX2 are enzymes responsible for the synthesis of important biological mediators called prostanoids and play divergent roles. In the gastrointestinal tract, COX-1 maintains the normal lining of the stomach. The enzyme is also involved in kidney and platelet function. Cyclooxygenase-2 (COX-2) is primarily present at sites of inflammation. Recent studies have demonstrated high COX-2 levels in several types of cancers (Eberhart et al., 1994), including skin cancer (Buckman et al., 1998; Muller-Decker, 2011; An et al., 2002; Smith et al., 2012). In fact, immunostaining of biopsies from SCCs show more intense staining for COX-2 protein, compared to normal skin (Buckman et al., 1998). In line with our results, Wilgus *et al* showed that topical treatment with a specific COX2 inhibitor (Celecoxib) prevented the formation of papillomas after continuous exposure to UV-B light (Wilgus et al., 2000; Wilgus et al., 2003). These results indicate that inhibition of inflammation could be therapeutically beneficial to prevent the development of cutaneous SCCs.

In addition, CD4 T cell infiltrates were found in human SCC, but not BCCs as assessed by CD4 immunostaining. Interestingly, 70% of the SCCs expressing high levels of c-FOS showed infiltration of CD4 T cells. This suggests that expression of c-Fos in human SCC could also promote infiltration of CD4 T cells. Further analyses should be performed to assess whether the presence of these cells could be used as a prognostic

marker in cutaneous SCCs. In contrast to our results, where we propose a tumor-promoting role of the CD4 T cell population, it is widely accepted that immunocompromised patients that undergo organ transplantation are prone to develop cutaneous SCCs (Muellenhoff and Koo, 2012). These patients are commonly treated with Calcineurin inhibitors such as cyclosporine A (Costanzo, 2001). Interestingly, recent studies have indicated that the effect of these inhibitors may not only impact on T lymphocytes, but may also have an impact on other cell types such as keratinocytes. Wu and colleagues demonstrated that genetic and pharmacological suppression of calcineurin/NFAT function promoted tumor formation in mouse skin and in xenografts by activating expression of ATF-3 in keratinocytes (Wu et al., 2010). Further evidence also suggests that cyclosporine A can also inhibits DNA repair thus increasing the skin cancer risk (Kuschal et al., 2012). However, other immunesupressive drugs are being tested to avoid using cyclosporine A inhibitors (Euvrard et al., 2012).

Therefore, my studies have helped to better understand the contribution of the CD4 T cells in the development of preneoplastic lesions and SCCs in the skin and suggest that the use of antiinflammatory drugs may be beneficial to prevent development of skin cancer.

Figure 31.



Model summarizing the cascade of events occurring upon sustained expression of expression in the epidermis.

Conclusions

Conclusions for objective #1

1. Inducible *c-fos* expression in the basal layer of the epidermis of c-Fos$^{Ep\text{-}tetON}$ and c-Fos$^{Ep\text{-}tetOFF}$ mice leads to the development of preneoplastic lesions. These lesions are characterized by increased proliferation, hyper- and parakeratosis, cell atypia and infiltration of inflammatory cells.
2. Inducible expression of *c-fos* in the basal layer of the epidermis promotes keratinocyte proliferation *in vivo,* but not *in vitro.* Moreover, epidermal differentiation is partly delayed, since Notch signaling pathway activation is impaired.
3. Upon inducible *c-fos* expression in the epidermis, transient and chronic infiltration of $Gr1^+$ and $CD4^+$ T cells, respectively, is observed by Flow Cytometry analyses. Moreover, FACS sorting of CD4 T cells from backskin of c-Fos$^{Ep\text{-}tetON}$ mice revealed dominant expression of the pro-inflammatory cytokine IL-22.
4. Inducible expression of *c-fos* is not required to sustain the preneoplastic lesions since upon *c-fos* switching off, preneoplastic lesions regress and the epidermis is restored to a homeostatic condition.
5. Inducible *c-fos* expression after a single dose of DMBA is sufficient to promote development of invasive SCCs in c-Fos$^{Ep\text{-}tetON}$ and c-Fos$^{Ep\text{-}tetOFF}$ mice.
6. Tumor burden, number and size of invasive SCCs in c-Fos$^{Ep\text{-}tetON}$ were reduced when the DMBA/c-Fos treatment was combined with the application of a COX1/COX2 inhibitor.

Conclusions for objective #2

7. Genome-wide expression analyses using primary c-Fos^{tetON} keratinocytes identified *mmp10* and *s100a7a15* as two putative target genes of c-Fos. Furthermore, chromatin immunoprecipitation analyses revealed direct binding of c-Fos to *mmp10* and *s100a7a5* promoters thus indicating that they are direct transcriptional targets of c-Fos/AP-1. MMP10 and S100a7a15 proteins are likely responsible for immune cell recruitment.
8. Inhibition of MMP10 signaling ameliorates the development of the preneoplastic lesions upon inducible *c-fos* expression in c-Fos$^{Ep\text{-}tetON}$ mice.

9. Depletion of mature B and T cells by crossing c-Fos$^{Ep\text{-}tetON}$ mice to a Rag1 deficient background prevents the development of preneoplastic lesions.

Conclusions for objective #3

10. Immunostainings in paraffin embedded human samples demonstrated that 80% of human SCCs expressed c-FOS protein compared to helathy skin whereas in BCCs no c-FOS expression was detected. Moreover, 70% of the SCCs showed coexpression of both c-FOS and MMP10. However, no coexpression of c-FOS with S100A7 was observed. Furthermore, 70% of the SCCs expressing high levels of c-FOS presented CD4 T cell infiltrates.

Conclusiones

Conclusiones del objetivo #1

1. La expresión inducible de *c-fos* en el capa basal de la epidermis en los ratones c-Fos$^{Ep\text{-}tetON}$ y c-Fos$^{Ep\text{-}tetOFF}$ promueve el desarrollo de lesiones preneoplásicas. Estas lesiones se caracterizan por ser altamente proliferativas, presentar hiper- y paraqueratosis, atipia celular e infiltración de células inmunes.
2. La expresión inducible de *c-fos* en la capa basal de la epidermis promueve proliferación en los queratinocitos *in vivo*, pero no *in vitro.* Además, la diferenciación epidérmica se retrasa parcialmente dado que la vía de señalización de Notch está inactivada.
3. Los ensayos de citometría de flujo revelan infiltración transitoria de células Gr1^{+} e infiltración crónica de linfocitos T CD4^{+} en la piel de los ratones c-Fos$^{Ep\text{-}tetON}$. Además, el aislamiento de los linfocictos T CD4 de la piel de los ratones c-Fos$^{Ep\text{-}tetON}$ mediante FACS reveló expresión dominante de la citoquina IL-22.
4. Las lesiones preneoplásicas desarrolladas en la piel de los ratones c-Fos$^{Ep\text{-}tetON}$ revierten y la epidermis vuelve a una situación homeostática al apagar la expresión inducible de *c-fos.*
5. La expresión inducible de *c-fos* después de la aplicación de DMBA en la piel de los ratones c-Fos$^{Ep\text{-}tetON}$ y c-Fos$^{Ep\text{-}tetOFF}$ promueve el desarrollo de carcinomas epidermoides invasivos en los ratones.
6. El uso del inhibidor de las enzimas COX1/COX2 en combinación con la aplicación de DMBA y la expresión inducible de *c-fos* reduce la carga tumoral así como el número y tamaño de los carcinomas epidermoides en los ratones c-Fos$^{Ep\text{-}tetON}$.

Conclusiones del objetivo #2

7. Análisis masivos de expresión génica utilizando queratinocitos primarios aislados de ratones c-Fos^{tetON} identificaron dos genes cuya expresión incrementó al inducir expresión de *c-fos.* Además, análisis de immunoprecipitación de cromatina revelan la unión de c-Fos a los promotores de *mmp10* y *s100a7a15* indicando que c-Fos controla transcripcionalmente la expresión de estos dos genes.

8. La inhibición de la actividad de la proteína MMP10 reduce el desarrollo de las lesiones preneoplásicas al inducir la expresión de *c-fos* en la capa basal de la epidermis en los ratones c-Fos$^{Ep\text{-}tetON}$.
9. La depleción de linfocitos maduros B y T al cruzar el ratón c-Fos$^{Ep\text{-}tetON}$ con un ratón knock-out de Rag1 previene el desarrollo de las lesiones preneoplásicas.

Conclusiones del objetivo #3

10. Las inmunotinciones en tejido embebido en parafina demuestran que el 80% de los carcinomas epidermoides humanos analizados expresan altos niveles de c-FOS comparado con la piel adyacente al tumor mientras que en los carcinomas basales no se detectó expresión de c-FOS. Además, el 70% de los carcinomas epidermoides coexpresan ambas proteinas, c-FOS y MMP10 pero no se obervó coexpresión entre c-FOS y S100A7. Finalmente, el 70% de los carcinomas epidermoides que expresan niveles altos de c-FOS presentaban infiltración de linfocitos T CD4^{+}.

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Appendix

Table A

AP-1 Protein	Phenotype	Affected organs/cells	Reference
		TRANSGENIC	
H2Kb-Jun	None	H2Kb-Jun	(Grigoriadis et al., 1993)
UBC-JunB	Increased bone mass	Thymus, CD4 thymocytes	(Schorpp et al., 1996)
CD4-JunB	Enhanced Thelper cell 2 maturation	Thymus, CD4 thymocytes	(Li et al., 1999)
UBC-JunD	Peripheral T cells and B cells reduced	Lymphocytes	(Meixner et al., 2004)
H2Kb-Fos	Osteosarcoma	Bone, osteoblasts	(Grigoriadis et al., 1993)
H2Kb-c-Fos/Rsk-2$^{-/y}$	Reduced osteosarcoma	Bone, osteoblasts	(David et al., 2005)
H2Kb-FosB	None	Bone	(Grigoriadis et al., 1993)
TCRb-ΔFosB	Impaired T cell differentiation	Thymus, immature thymocytes	(Carrozza et al., 1997)
NSE-DFosB	Osteosclerosis	Bone, osteoblasts	(Sabatakos et al., 2000)
H2Kb-Fra-1	Osteosclerosis	Bone, osteoblasts	(Jochum et al., 2000)
CMV-Fra-2	Increased bone mass, fibrosis	Bone, internal organs, skin	(McHenry et al., 1998)
H2Kb-Fra-2	Lung fibrosis, Osteosclerosis	Lung, Bone, osteoblasts	(Eferl et al., 2008)
		KNOCK-OUT	
c-Jun	Embryonic lethal at E12.5	Liver, heart, neural crest	(Hilberg et al., 1993; Johnson et al., 1993; Johnson et al., 1993)
JunB	Embryonic lethal at E10	Extraembryonic tissues	(Schorpp-Kistner et al., 1999)
JunD	Male sterility	Testis, spermatides	(Thepot et al., 2000)
c-Fos	Osteopetrosis	Bone, osteoclasts	(Wang et al., 1992)
FosB	Nurturing defect	Brain, hypothalamus	(Gruda et al., 1996; Brown et al., 1996)
Fra-1	Embryonic lethal at E9.5	Exraembryonic tissue	(Schreiber et al., 2000)
Fra-2	Lethal after birth	Bone, osteoclasts	(Eferl et al., 2007)
		CONDITIONAL	
Alfp-cre c-Jun	Liver regeneration defect	Liver, hepatocytes	(Behrens et al., 2002)
Alfp/Mx-cre c-Jun +DEN	c-Jun required for liver initiation tumorigenesis	Liver, hepatocytes	(Min et al., 2012)
Col2a1-cre c-Jun	Scoliosis	Bone, notochonrdal cells	(Behrens et al., 2003)
Nestin-cre c-Jun	Axonal regeneration defect	Central nervous system, motoneurons	(Raivich et al., 2004)
MORE-cre JunB	Osteopenia	Bone, osteoclasts, osteoblasts	(Kenner et al., 2004)
MORE-cre Fra-1	Osteopenia	Bone, osteoblasts	(Eferl et al., 2004)

Nestin-cre Fos	Learning defects	Bran, hippocampal neurons	(Fleischmann et al., 2003a)
K5-cre c-Jun	Eyes open at birth, reduced skin tumors	Keratinocytes	(Zenz et al., 2003)
K5-cre c-Jun, K5-SOS			(Zenz et al., 2003)
K5-cre Fra-2	Epidermal hyperplasia and inflammation	Keratinocytes	Wurm, unpublished
K5-cre JunB	Myeloproliferative disease, bone loss, epidermal hyperplasia and inflammation	Keratinocytes	(Meixner et al., 2008)
K5-cre c-Fos	None	Keratinocytes	(Guinea-Viniegra et al., 2012)
K5-cre Fos, K5-SOS	Reduced papilloma size with increased differentiation	Keratinocytes	(Guinea-Viniegra et al., 2012)
K5-cre JunB, c-Jun	Systemic inflammatory response with cachexia	Keratinocytes	(Guinea-Viniegra et al., 2009)
	INDUCIBLE		
K5-creER^{T}JunB + Jun	Psoriasis-like disease	Skin, joints, keratinocytes	(Zenz et al., 2005)
K5-creER^{T}Fos; K5 SOS	Reduced papilloma size	Keratinocytes	(Guinea-Viniegra et al., 2012)